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GRANT NUMBER DAMD17-96-1-6096

TITLE: Nitric Oxide in Mammary Tumor Progession

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (July 1, 1998 - June 30, 1999)

4. TITLE AND SUBTITLE

Nitric Oxide in Mammary Tumor Progression

5. FUNDING NUMBERS

DAMD17-96-1-6096

6. AUTHOR(S)

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The University of Western Ontario
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

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13. ABSTRACT *(Maximum 200* Nitric Oxide (NO) is a potent bioactive molecule produced in the presence of endothelial (e), neuronal (n) and inducible (i) types of NO synthase (NOS) enzymes. We have shown that NO was responsible for IL-2 therapy-induced capillary leakage because of iNOS induction in various tissues by IL-2 therapy. We also found that tumor-derived NO promoted mammary tumor progression in C3H/HeJ mice. eNOS expression by tumor cells was positively correlated with metastasis in spontaneous C3H/HeJ mammary tumors and transplants of two clonal derivatives of a spontaneous tumor differing in metastatic phenotype: highly metastatic C3L5 and weakly metastatic C10 cell lines. These cell lines also exhibited a parallel difference in invasiveness *in vitro* and growth rates as well as angiogenic abilities *in vivo*. A causal relationship between NO production by the tumor cells and invasive, migratory and angiogenic abilities was demonstrated. Invasion stimulation by NO was mediated by an upregulation of matrix degrading enzyme matrix metalloprotease (MMP)2 and a downregulation of MMP inhibitors TIMP-2 and TIMP-3. Thus tumor-derived NO promoted mammary tumor progression by stimulating tumor cell migration, invasiveness and angiogenesis, indicating a valuable therapeutic role of NOS inhibitors for blocking multiple steps in tumor growth and metastasis.

14. SUBJECT TERMS

Breast Cancer, Nitric Oxide, invasion, metastasis, C3H/HeJ mammary tumor, Angiogenesis, IL-2 therapy

15. NUMBER OF PAGES

95

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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5. INTRODUCTION

This project has been designed to explore the role of nitric oxide (NO) in mammary tumor progression, using a C3H/HeJ mouse mammary tumor model developed in our laboratory. This model employs spontaneous tumors as well as their clones which vary in their ability for spontaneous metastasis.

Biology of NO

Following the discovery (1) that NO accounts for the full biological activity of a factor initially named "endothelium-derived relaxing factor" (2), produced by endothelial cells and causing vasodilation, research on the biology of NO has grown exponentially for many years. This molecule has since been shown to be produced by many other cells in the body, providing additional physiological functions such as inhibition of platelet aggregation, modulation of neurotransmission and mediation of cytotoxic function of macrophages against microbes, parasites and tumor cells (3-8). Sustained high levels of NO produced at the sites of inflammation can also mediate pathological injuries (9).

NO is produced by the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes known as NO synthases (NOS). Three isoforms of NOS have been identified so far: endothelial type or eNOS is a constitutive form present in endothelial cells, myocardial cells and other cells inclusive of certain tumor cells; neuronal type or nNOS is also a constitutive form present in the central nervous system neurons, cells of the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells as well as in tumors of the central nervous system; inducible type or iNOS is usually induced by certain inflammatory cytokines (e.g. IFN-γ, TNF-α) or bacterial products (e.g. LPS) in macrophages, hepatocytes, chondrocytes, endothelial cells and certain tumor cells (10-14). The constitutive forms are Ca^{++} and calmodulin-dependent whereas the inducible form is Ca^{++} and calmodulin-independent. Genes for all the isoforms have been cloned in numerous species (15,16) and disrupted in mice to show that none of the disruptions were embryo-lethal but had pathological effects consistent with known biological functions of NO. For example, eNOS knockout mice are hypertensive (17) because of the loss of vaso-relaxant function of NO; iNOS knockout mice are susceptible to infection and show poor macrophage cytotoxicity against parasites and tumor cells (18), consistent with NO-mediated macrophage defense; nNOS knockout mice (19) show hypertrophic pyloric stenosis, consistent with NO-mediated relaxation of pyloric sphincter muscle. nNOS -deficient males, in addition, show abnormal sexual behavior (20) because of aberrant neurotransmission.

NO is a free radical capable of crossing the cell membrane and reacting with other molecules. Most physiological functions of NO are mediated by increases in intracellular cGMP (21,22), whereas antibacterial, antiparasitic and antitumor functions of macrophage-derived NO

have been ascribed to the inhibition of mitochondrial respiration and DNA synthesis in target cells (23).

Constitutive production of NO occurs in cells at low to moderate levels, and the resulting bioactivity is short lived (T½ = few seconds) and short-range in nature. On the other hand, induced production of NO can be sustained at high local levels for a longer duration if the inducer molecules, e.g. inflammation-associated cytokines are produced in a protracted manner. This often leads to pathological consequences, resulting from NO reaction products. NO reacts with molecular oxygen, transition metals and superoxide to form intermediates which can cause cellular injury. For example, NO reacts with superoxide to make peroxynitrite, which can cause DNA damage (24).

Role of NO in tumor progression

It has been recognized for some time that chronic NO production is genotoxic and thus potentially carcinogenic (24). Recent studies, including our own (25) have revealed that tumor or host-derived NO can profoundly influence tumor progression in a positive or negative manner depending on the circumstances, and that in a large panel of well-established tumors, which have been examined so far, NO usually promotes tumor progression. Elevated serum NO levels have been observed in many cancer patients (26) indicating that tumor cells or host cells serve as the additional source of NO in these patients. A high expression of active NOS enzymes in tumor cells (27,28,31-33), endothelial cells in tumor vasculature (28) or tumor-infiltrating macrophages (29,30,32) has been positively correlated with the degree of malignancy in human cancers involving a large number of tissues: cancers of the reproductive tract (uterus, ovary) (27), central nervous system tumors (28), breast cancer (29), gastric cancer (30), cancer (squamous cell carcinomas) of the head and neck (31), prostate cancer (32) and lung cancer (33). However, the underlying mechanisms remain unexplored. Unexpectedly an inversion of this relationship was reported for human colonic tumors (34,35); this finding was in contrast with another study reporting that many human colon cancer cell lines exhibited significant NOS activity (36). This discrepancy has finally been resolved in a recent study (37) showing that the highest expression of active iNOS was noted in human colonic adenomas prior to their progression into carcinomas, consistent with the hypothesis that this promoted the transition of adenomas into carcinomas by a stimiulation of angiogenesis. A positive correlation between NOS expression or NO production and tumor progression has also been detected in experimental tumor models in the mouse (38) and the rat (39).

A direct evidence for a stimulatory role of NO in tumor progression came from our own findings in a murine mammary adenocarcinoma model that treatments with either of two NOS inhibitors N^G-methyl-L Arginine (NMMA)(40)and N^G-nitro-L-arginine methyl ester (L-NAME) (41) reduced the growth of the primary tumors and their spontaneous lung metastases in mice

transplanted with the C3L5 mammary tumor line (reviewed in 25). Similar findings were reported with L-NAME therapy in a rat colonic adenocarcinoma model (39). In support of these results, engineered expression of iNOS in a human colonic adenocarcinoma line resulted in an increased growth rate and vascularity of tumors following transplantation in nude mice (42). In contrast with these results, engineered overexpression of iNOS in an iNOS deficient murine melanoma line (43,44) or a human renal carcinoma line (45) suppressed tumorigenic and metastatic ability of tumor cells *in vivo* because of NO-mediated cytostasis and apoptosis (43,44). Two explanations may be offered for these apparently conflicting results: First, very high NO levels (such as those produced by the iNOS-transduced murine melanoma line) (43,44) can be detrimental to tumor cell survival; for example the iNOS-overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* (44). Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis because of their genetic makeup. For example, it has been suggested that the functional status of the tumor suppressor gene p53 dictates susceptibility (if functional) or resistance (if non-functional) to NO-mediated cytostasis or apoptosis (46,47). This suggestion was based on the following findings: iNOS transfected tumor cell lines fell into two distinct categories. Those expressing functional wild type p53 were vulnerable to NO-mediated cytostasis because of an accumulation p53 protein induced by endogenous NO (46,47). On the other hand, tumor cells in which p53 gene was lost or mutated not only withstood the deleterious effects of endogenous NO, but also exhibited faster growth and vascularity when transplanted *in vivo* (47). Since p53 mutation occurs in nearly half of human cancers (48), it was hypothesized that NO would facilitate tumor progression in a large proportion of well-established human tumors (47). We hypothesize that during the clonal evolution of tumors *in vivo*, high NO producing clones susceptible to NO-mediated injury are deleted and selected against those which are genetically resistant to NO-mediated injury and capable of utilizing NO to their advantage for expression of an aggressive phenotype (25). Loss of functional p53 gene may represent one of many genetic changes which can possibly result in the above phenotype. Further studies are needed to identify other genotypic markers in tumors for susceptibility or resistance to NO-mediated injury, so that the information can be utilized in therapeutic designs.

C3H/HeJ mammary tumor model employed in the present project.

Details of this model are provided in Appendix 1. In brief, this model is a combination of spontaneous C3H/HeJ mammary tumors and some of their clonal derivatives produced in our laboratory. Approximately 90% of retired breeder females of this mouse strain spontaneously develop invasive mammary adenocarcinomas with a pseudoglandular architecture, all of which metastasize to the lungs. Tumor development is due to insertional mutagenesis of certain cell growth-regulating loci resulting from the integration of the proviral form of the mouse mammary tumor virus (MMTV) in the developing mammary tissue of mice receiving the virus via mother's milk. Approximately 39% of human breast cancer specimens express a 660 bp sequence of the

MMTV envelop gene (49), the epidemiological significance of which remain to be identified. This finding and the similarity in histological features and metastatic behaviour suggest that C3H/HeJ spontaneous mammary tumors may represent the closest model for the human breast cancer, in particular, the familial form. We have derived two clonal lines, C3L5 and C10, grown from a spontaneous mammary tumor-derived line T58. The metastatic phenotype for C3L5 is high, for C10 is low, and for T58 is intermediate, based on the number of spontaneous lung metastases from subcutaneously transplanted tumors.

Preliminary data provided in the original grant application and substantiated further in the last two annual reports revealed that spontaneous C3H/HeJ primary tumors expressed eNOS protein (based on immunocyto-chemistry) in a heterogenous manner in tumor cells, whereas their metastases in the lungs were uniformly and strongly positive for eNOS (25). This finding suggested that eNOS bearing cells in the primary tumor were more prone to metastasis. This suggestion was strengthened by the findings that C3L5 cells (highly metastatic) were strongly positive for eNOS *in vitro*, as well as *in vivo* both at primary and metastatic sites (25). In addition, iNOS was inducible in C3L5 cells when cultured with IFN-γ and LPS (25). In contrast, C10 cells (poorly metastatic) were weakly positive for eNOS, and the expression was heterogenous. These findings, combined with our observations (25,40,41); that two NOS inhibitors NMMA and L-NAME reduced the growth of C3L5 primary tumors as well as their spontaneous lung metastases, led us to hypothesize that tumor-derived NO promoted tumor progression in this mammary tumor model. A large component of the current project is to validate this hypothesis and to identify the mechanisms underlying NO-mediated promotion of tumor progression in this model.

Role of NO in "capillary leak syndrome"

We discovered that capillary leak syndrome (characterized by fluid leakage from the capillaries into tissue spaces, various organs and body cavities), a life-threatening side effect of interleukin-2 (IL-2) based cancer immunotherapy, is due to the increased production of nitric oxide (40,41,50). This was shown by (a) a positive correlation of NO levels in the serum and the body fluids with the severity of IL-2 therapy-induced capillary leakage in healthy and tumor-bearing mice, and (b) an amelioration of this capillary leakage by chronic oral administration of NOS inhibitors NMMA and L-NAME (see ref. 52 for a comprehensive review).

Unexpectedly, we also observed that additional therapy with NOS inhibitors improved antitumor/antimetastatic effects of IL-2 therapy (40,41). This finding led to the suggestion that NO induction by IL-2 therapy interfered with antitumor effects of IL-2 therapy. We tested this hypothesis by investigating the effects of addition of L-NAME on IL-2 induced generation of lymphokine activated killer (LAK) cells *in vivo* and *in vitro* in healthy and tumor bearing mice (53). Results revealed that inhibition of NO production *in vivo* or *in vitro* by addition of

L-NAME to IL-2 therapy or IL-2 induced lymphocyte activation *in vitro* caused a substantial enhancement of LAK cell activation. In other words, IL-2 induced NO production interfered with optional LAK cell activation which can be abrogated with NOS inhibitors (53).

A minor component of the current project was to (a) identify the cellular source of NO induced by IL-2 therapy, (b) identify the nature of structural damage to the lungs of mice suffering from IL-2 induced pulmonary edema and pleural effusion, and (c) examine the effects of L-NAME therapy on the above parameters. Results of these studies have been published (51). In brief, IL-2 therapy led to high levels of iNOS protein expression and activity in the tissues of the anterior thoracic wall in accompaniment with pleural effusion. There was structural damage to the lungs (alveolar epithelium and interstitial tissue) and its capillaries by IL-2 therapy, which were mitigated by L-NAME therapy. L-NAME therapy abrogated IL-2 induced rise in iNOS activity but not the expression iNOS protein in the tissues.

6. BODY OF THE PROGRESS REPORT

Overall Hypothesis: Tumor derived NO promotes C3H/HeJ mammary tumor progression and metastasis.

Overall Objectives:

(1) To validate the hypothesis of the stimulatory role of NO in mammary tumor progression by further correlation of eNOS expression with metastasis, and investigating the effects of down-regulating eNOS gene on tumor cell behaviour *in vitro*, e.g. migration and invasiveness, and *in vivo*, e.g. tumor growth, angiogenesis and metastases.

(2) To identify mechanisms of NO-mediated stimulation of tumor progression by investigating the role of NO in tumor cell proliferation, migration, invasiveness and tumor-induced angiogenesis.

Our assessment of overall progress in relation to the statement of objectives

Task 1 **Relationship between NOS expression and tumor progression/ metastasis:** Progress has matched with our expectations in components 1a and 1b. The molecular biology components have continued to be frustrating. This was initially because of our failure to knockout the eNOS gene in C3L5 cells, evidently because of increased number (3.6) of gene copies in these cells. Last year we adopted the antisense RNA approach to downregulate eNOS. We succeeded in obtaining low or nonexpressing clones more than once, however, all of them proved to be unstable and reverted to the expressor phenotype. Currently we are applying antisense oligonucleotides to achieve downregulation of a shorter duration.

Task 2 **Identification of mechanisms of tumor progression by NO.** Although this task was initially assigned to Year II onwards, we have achieved significant progress in this area within Years I, II and III and nearly completed our goals. We are designing some newer experiments on the basis of our current findings (see later).

Task 3 **Mechanisms underlying IL-2 induced capillary leakage and interference with antitumor effects of IL-2 therapy by IL-2-induced NO.** Although this task was initially assigned to Year III onwards, we have completed our goals in this area. We are continuing some newer experiments designed on the basis of newer knowledge. (See later).

Record of Research findings during the current year.

Task 1 Relationship between NOS expression and tumor progression and metastasis.

(a) Relationship between the expression of NOS protein and tumor growth and metastasis.

(i) Spontaneous C3H/HeJ mammary tumors.

It took us long to accrue data on a sufficient number of spontaneous tumors which develop slowly during the lifetime of the female retired breeder C3H/HeJ mice (54). We completed the examination of the relationship of the level of eNOS protein expression to tumor growth and metastasis in 26 spontaneous tumors and their metastatic foci. Because of large amounts of necrosis in 6 tumors (which causes nonspecific staining of necrotic cells), they were excluded from the analysis. Materials, methods and the results based on 20 tumors are detailed in the submitted manuscript, Appendix 1.

In summary, spontaneous tumors at the primary sites showed heterogenous eNOS expression in tumor cells. Irrespective of tumor growth rates (whether fast, intermediate or slow) a mixture of strongly eNOS positive (40-70%) or completely eNOS negative cells (30-60%) (Appendix 1, Figure 2A) were observed, however, the proportion of positive cells were higher in poorly differentiated areas than in differentiated areas of tumors showing pseudoacinar arrangement of tumor cells (Appendix 1, Figures 2A, 2B). In contrast, virtually all tumor cells at the sites of lung metastasis were strongly and homogenously eNOS positive (Appendix 1, Figure 2C). iNOS expression was restricted to a subset of macrophages within the primary tumors or the tumor stroma (Appendix 1, Figure 2D) as well as the metastatic sites. These results provide a strong validation of our preliminary data (25) suggesting a positive association between eNOS expression and tumor growth and metastasis, leading to the hypothesis that eNOS expression provides tumor cells with a selective advantage for growth and metastasis.

Some of the above data were presented at the last meeting of the American Association of Cancer Research (Appendix 2) and also the "Reasons for Hope" Conference in Breast Cancer Research sponsored by the Canadian Breast Cancer Research Initiative this year (Appendix 3).

(ii) C3L5 (highly metastatic) and C10 (weakly metastatic) cell lines and their transplants.

(a) The data are detailed in Appendix 1, and were presented at two meetings (Appendices 2 and 3).

In summary, subcutaneous transplants of C3L5 cells grew faster at the primary sites and produced a larger number of spontaneous lung metastasis than those of C10 cells during the same time span (Figure 1, Appendix 1). Weakly metastatic C10 cells expressed low levels of eNOS *in vitro* in a minor proportion of cells, as compared to the highly metastatic C3L5 cells which expressed high eNOS levels in nearly every cell. Similar differences in eNOS protein expressin were observed *in vivo* only at the primary tumor site (Figures 2E and G, Appendix 1), however their lung metastasis were equally and strongly positive for eNOS (Figures 2F and 2H, Appendix 1). iNOS expression was undetectable in either cell line, but noted in a subset of both cell lines *in vitro* only when cultured in the presence of IFN-γ + LPS. C3L5 and C10 tumor cells grown *in vivo* were iNOS negative both at primary and metastatic sites; only a subset of macrophages at either site expressed iNOS. These results were objectively validated by quantitative image analysis (Figures 3A and 3B, Appendix 1), and supported the hypothesis of a causal relationship of eNOS expression to tumor growth and metastasis.

(b) Attempts to investigate biological alterations of murine mammary adeno-carcinoma cell line (C3L5) by downregulation of eNOS gene expression.

Because of the presence of very high copy number of eNOS gene in C3L5 breast cancer cells, we were unable to knock-out eNOS gene in these cells. Therefore, we have been trying to knock-down this gene by stable tranfection of the cells with antisense RNA. We subcloned a 4Kb full length cDNA of human eNOS to an expression vector pCR^{TM}-3 at both sense and antisense orientations, transfected using Lipofectamine, and selected the transfected cells using G418. As documented in last year's report, we obtained some eNOS downregulated clones. We maintained these clones in culture for two months. Unfortunately, they began to re-express eNOS gene and protein. This disappointment happened twice. This phenomenon is not unusual in antisense RNA (AS-RNA) field. (55) Although only a few publications (56, 57) address the failure of AS-RNA to suppress expression of targeted genes, it is well known that there are a lot of disappointments in this field which remain undocumented. The cells might have increased the efficiency of other regions of the construct viz. SV40 termination site so that the read-through transcription of the antisense RNA is reduced (55). However, there could be many other unknown factors which might affect biological actions of AS-RNA. In fact, general principles governing the regulation of AS-RNA are yet to be established. That is why construction of a series of transformation vectors with different portions of the gene (with different sizes) is recommended, because different portions of the gene may vary in their ability to inhibit gene expression (55). Furthermore, there must be an excess of AS-RNA

over mRNA in the nucleus, i.e. if a particular AS sequence is titrated out by other mRNAs, there might be less inhibition of the target mRNA. This may be a particuilar problem in cells with increased number of gene copies. Although we did not try to find out the reasons of the failure, we intend to make at least two more AS constructs with two different fragments of eNOS gene (one of which will be derived from the tail region of the gene), and the constructs will be used at different concentrations to transform C3L5 cells.

We have also taken antisense oligonucleotide knock-down strategy to downregulate eNOS gene expression in C3L5 cells. It is clear from the literature that not all antisense oligonucleotide sequences can downregulate expression of target proteins. Many researchers believe that phosphothioate (PS) oligodeoxy-nucleotides inhibit protein expression by blocking the ribosome as it moves along mRNA. That is why the dogma is that the design of oligonucleotides in the vicinity of AUG (start codon) site on the mRNA are most active. But because almost all PS oligos function as antisense molecules by forming duplex with target mRNA and then serves as substrate for RNase H, Dr. Nic Dean of ISIS Pharmaceutical recommends to design several oligo sequences throughout the mRNA (58). In fact, his group has shown in many instances (58-60) that PS-oligos designed in the vicinity of AUG (start codon) site have very little or no potential to inhibit target protein expressions, whereas the maximal ability to inhibit target protein expression was shown by PS-oligos designed from some other regions of the mRNA. Furthermore, the phosphothioate modifications of the oligodeoxy-nucleotides, although effective, have some limitations. Their main limitation is that they are metabolised in cells over time, leading to almost total loss of activity over 48-72 h period. Dr. Nic Dean's group has shown that the 2'-O-(2-methoxy) ethyl (2'-MOE) modification of oligodeoxynucleotide result in dramatic enhancement in the ability of the sequence to hybridize to a target mRNA and nuclease resistance when compared with PS-oligos (61). Therefore, we requested him to synthesize 2'-MOE modified antisense murine eNOS oligodeoxynucleotides for us. He has kindly synthesized 22 different oligos designed from different regions of the eNOS mRNA for us, the sequences of which remain blinded to us. We are in the process of using these oligos in our cell culture in order to screen an active sequence. We shall use lipofectin as oligo uptake enhancer. 100-400 nM concentrations of each oligo will be mixed with appropriate concentrations of lipofectin (5μl lipofectin/ml/100 nm oligo). These mixtures will be added to the cells when they are 70-80% confluent. The cells will be allowed to incubate for 4 hrs with occasional swirl. The lipofectin solution will then be removed and replaced with media. The cells will be allowed to incubate overnight, NO concentrations in the medium and eNOS mRNA in the cells (by

RT-PCR) will be measured. Once we identify the best sequence, Dr. Dean will send us more of the compound along with appropriate controls for our functional assays like migration, invasion and angiogenesis.

Our main effort will be focussed on the AS-oligo study rather than AS-RNA study. AS-RNA study (stable transfection) will be our second priority. This is mainly because we already have substantial results with our naturally occurring low eNOS expressing C10 cell line clonally derived from the same parental tumor from which the high eNOS expressing C3L5 line was derived.

Task 2 **Identification of mechanisms underlying NO-mediated promotion of tumor progression.** We hypothesized that tumor-derived NO facilitates tumor progression and metastasis by **(a)** promoting tumor cell migratory ability, **(b)** promoting tumor cell invasive ability and **(c)** promoting tumor-induced angiogenesis which is critical for the growth of solid tumors. We had already shown that tumor-derived NO exerted no influence on tumor cell proliferation *in vitro*. Others have shown that tumor-derived NO promotes tumor blood flow and microcirculation which can indirectly promote tumor growth (62-64).

(a) Effects of tumor-derived NO on *in vitro* migratory ability of C10 and C3L5 tumor cells.

Since migratory ability is an essential component of cellular invasiveness and metastasis, we examined the role of NO on the migration of the two mammary tumor cell lines differing in eNOS expression and metastatic phenotype. First, we compared the migration kinetics of the two cell lines using a transwell migration assay detailed in Appendix 1. Second, we examined the migratory ability of each cell line after treatment with the NOS inhibitor L-NAME in the presence or absence of excess L-arginine (the natural substrate for NOS, which should compete with L-NAME) under conditions detailed in Appendix 1, in order to identify the contributory role of NO which was measured in the medium under identical conditions.

Results (detailed in Appendix 1) revealed that the two cell lines did not differ significantly in their migratory abilities (Figure 4A, appendix 1), however, migration of both cell lines were inhibited in the presence of L-NAME in a dose-dependent manner and restored in the additional presence of excess L-arginine (Figure 5A, Appendix 1). These treatments produced correspondingly similar effects on the NO production by these cells (Figure 6, Appendix 1). These results demonstrate that migration of both cell lines are stimulated by endogenous NO, in spite of differences in the level of NO production by these cells. Thus an

absence of any significant difference in the basal migration rates of the two cell lines is possibly explained by differential production of other migration-regulating molecule(s) by these cells. The above is the first demonstration of migration stimulation of tumor cells by tumor-derived NO. The underlying mechanisms of signal transduction remains to be investigated.

We wish to conduct additional experiments (not proposed earlier) to identify intracellular pathways of signal transduction responsible for NO-mediated stimulation of tumor cell migration, utilizing the high eNOS-expressing C3L5 mammary tumor cell line. It has been shown that most physiological functions of NO (e.g. vasorelaxation by endothelium derived NO) are mediated by stimulation of cyclic GMP (cGMP) (10-12). In addition, vascular endothelial growth factor (VEGF)-mediated angiogenesis, involving endothelial cell proliferation, migration and tube formation has been shown to be dependent on stimulation of endothelial NOS (i.e. NO production) followed by activation of mitogen activated protein (MAP) kinase pathway (65,66). We postulate that endogenous NO causes an increase in the level of cGMP leading to the activation of cGMP dependent protein kinase (G kinase), MAP kinase kinase (MAPKK; extra cellular regulated kinases or ERK 1 and 2) and MAP kinase, followed by the activation of the cellular motility apparatus. This hypothesis will be tested in C3L5 cells as follows:

(i) We shall measure cGMP levels with a radioimmunoassay in cell extracts (65) following treatment of cells with L-NAME (to block NO production) ± excess L-arginine (which will restore NO production). Similar experiment will be done following treatment with LPS + IFN-γ to stimulate NO production by induction of iNOS. Levels of NO (measured with Griess reaction) will be correlated with cGMP levels. Migration index of intact cells will be measured under identical treatment conditions.

(ii) Cause-effect relationship of cGMP or G-kinase stimulation to cellular migration will be tested by treatment of cells with soluble guanylate cyclase inhibitors LY83583 (65) (0.1-10μM) or 1H (1,2,4) oxadiazolo (4, 3-a) quinoxalin-1-one (ODQ) (0.1-10μM) (66), G-kinase antagonist R p-8-pCPT- cGMPS (1-10nm) (67) and G-kinase inhibitor KT583 (1-10nm) (68) at proven nontoxic doses (i.e. having no effect on cell viability).

(iii) Whether eNOS or iNOS-derived NO causes activation (phosphorylation) of MAPKK (ERK1 and 2) will be tested with Western immuno blots of phospho ERK 1 and 2 (66) in lysates of cells pretreated with L-NAME ± excess L-arginine, or LPS + IFN-γ, as described in **(i)**.

(iv) Cause-effect relationship of MAPKK activation to cell migration will be tested by treatment of cells with a MAPKK inhibitor PD98059 (0-100μM) at proven nontoxic doses. Simultaneously, the effects of the inhibitor on ERK 1,2 phosphorylation will be tested.

(v) Effects of treating cells with L-NAME (NOS inhibitor) or G-kinase antagonist/inhibitor (see **ii** above) on ERK 1 and 2 phosphorylation would indicate whether NOS and G-kinase are proximal to MAPKK in the signal transduction pathway.

(b) Effects of tumor-derived NO on invasiveness of mammary tumor cells.

(i) A comparison of *in vitro* invasiveness of highly metastatic (and high eNOS expressor) C3L5 with weakly metastatic (and low eNOS expressor) C10 cell lines: role of NO.

Materials, methods and results are detailed in Appendix 1. In brief, C3L5 cells invaded matrigel at a faster rate than C10 cells (Figure 4B, Appendix 1). Furthermore, L-NAME caused a dose-dependent inhibition of invasion in both cells, which was relieved in the presence of excess L-arginine (Figure 5B, Appendix 1) with concomittant restoration of NO production (Figure 6, Appendix 1), indicating that endogenous NO stimulated invasiveness of both cell lines.

(ii) Mechanism of NO-mediated stimulation of invasiveness, investigated with C3L5 cells.

As presented in last year's Progress Report, C3L5 cell invasiveness was shown to be dependent on endogenous NO (Appendix 4, published manuscript).

Endogenous (eNOS-derived) NO was shown to downregulate TIMP-2 and TIMP-3 mRNA expression. In addition, when iNOS was induced in C3L5 cells by LPS+IFN-γ *in vitro*, these cells became more invasive, and there was an additional upregulation of MMP-2. Thus NO-mediated promotion of invasiveness was due to an altered balance between MMP-2 and its inhibitors TIMPs 2 and 3 (for details, see Appendix 4).

(c) Effects of tumor-derived NO on tumor-induced angiogenesis.

(i) C3L5 tumor model

We have devised a novel tumor angiogenesis assay employing implants of **growth factor-reduced** matrigel, inclusive of tumor cells in the matrigel suspension (abstracted in meeting presentation, Appendix 5; detailed in

Appendix 6, manuscript in press). Matrigel implants alone had no angiogenic effect, whereas inclusion of tumor cells caused significant angiogenesis. The time course, geography and levels of angiogenesis were objectively measurable after Masson's trichrome staining and CD31 (endothelial cell marker) immunostaining of sections. Animals received L-NAME (NOS inhibitor) or D-NAME (inactive enantiomer of L-NAME used as controls). D-NAME was found to have no effect on basal angiogenesis. Both drugs were given continuously via osmotic mini-pumps, to evaluate the effects of NOS inhibition on tumor-induced angiogenesis measured at 14 days after implantation of matrigel suspended tumor cells. Histological evaluation of implants revealed that neovascularization initially started in the periphery of the implants with concurrent development of stroma. Developing tumors were then fed by secondary vessels growing from the stromal region. At later time points (e.g. 14 days), necrosis was evident in the deeper areas of tumors. It was found that L-NAME treatment caused a dramatic inhibition of angio-genesis both in the stroma as well as tumor tissue as compared to D-NAME, and also a relative reduction in viable tissue mass (stroma and tumor) and an increase in necrosis. Detailed results are presented in Appendix 6. These data show that NO is a key mediator of C3L5 tumor-induced angiogenesis, and that growth inhibitory effects of L-NAME on the primary tumor are partly mediated by reduced tumor-angiogenesis.

(ii) A comparison of C3L5 (high eNOS expressor) and C10 (low eNOS expressor) tumor models

Materials, methods and results are detailed in Appendix 1. In brief, in the matrigel implant assay, C3L5 cells were more angiogenic than C10 cells, as expected from their differences in eNOS expression (Figure 7, Appendix 1). Unexpectedly, however, L-NAME treatment did not significantly affect C10 tumor induced angiogenesis indicating that either a certain threshold of NO level was required for angiogenesis stimulation, or that a differential upregulation of other angiogenic factor(s) may have compensated for the L-NAME inhibition of angiogenesis in C10 cells.

We have discovered that C3L5 cells express VEGF, a potent angiogenic factor. This remains to be examined in C10 cells. VEGF-induced angiogenesis (endothelial cell proliferation, migration and tube formation) has recently been shown to be dependent on NO production in endothelial cells following eNOS activation (evidently due to increase in intracellular calcium), leading to stimulation cGMP and then activation of MAPK pathway (65,66). Thus, it is likely that VEGF expression by C3L5 cells is an additional tool for NO-mediated angiogenesis because of eNOS activation in tumor cells as well as endothelial cells. We shall test whether a

neutralization of VEGF activity (with a VEGF neutralizing antibody) in C3L5 cells *in vitro* causes a reduction in Ca++ dependent NO production by C3L5 cells in culture medium. These will be expected if VEGF activates eNOS in C3L5 cells. We shall also test whether this treatment causes a reduction in intracellular Ca++ level in C3L5 cells.

Task 3 Role of NO in IL-2 induced capillary leakage and mechanisms by which this NO- production compromises antitumor effects of IL-2 therapy.

This task, as initially proposed, was completed earlier and published (52). We showed that IL-2 therapy induced active iNOS in tissues contiguous with pleural effusion and the resulting NO overproduction caused structural damage to the lungs and its capillaries. These injuries were ameliorated with the NOS inhibitor L-NAME.

These results raised the following questions. Was the damage to the lungs and its capillaries due to a direct injury (structural damage and apoptosis) by NO, or injury by certain reaction product of NO? Recently, it has been reported that oxygen-free-radicals play a role in IL-2 therapy-induced capillary damage because it could be ameliorated with dimethylthiourea, a scavanger of oxygen-free-radicals (62). We hypothesise that formation of peroxynitrite, a potent endotheliotoxic molecule, due to a combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary leakage. Last year we started testing this hypothesis by immunostaining for nitrotyrosine in the lungs of mice suffering from IL-2 induced pulmonary edema. Since cytotoxicity due to peroxynitrite is reported to be due to nitration of tyrosine-residues of intracellular tyrosine-kinases to form nitrotyrosine, nitrotyrosine provides a good marker for peroxynitrite mediated cellular injury. Our expectation was that this marker should appear in tissues of IL-2 treated mice showing capillary leakage and diminish in mice treated with IL-2 in combination with the NOS inhibitor L-NAME.

Preliminary results on capillary leakage and nitrotyrosine:

Experiment 1

7-8 week old C3H/HeJ mice (n = 10/group) were either given **(a)** no treatment; **(b)** IL-2 therapy alone (15,000 Cetus units /0.1 ml given i.p. every 8 hrs., 10 injections); **(c)** IL-2 + L-NAME therapy and **(d)** IL-2 + D-NAME (inactive enantiomer of L-NAME) therapy. L-NAME or D-NAME were delivered via osmotic minipumps (as detailed in appendix 5). Twelve hours after the last IL-2 injection (day 4) mice were sacrificed under CO_2 anesthesia, and lungs were removed. One lung from each animal was used to measure wet/dry weight ratios to detect capillary leakage, and the other lung was split into two, one half was cryopreserved, and the other half was paraformaldehyde-fixed, to make frozen and paraffin-embedded sections for

exposure of sections to a rabbit polyclonal antibody (Upstate Biotechnology) against nitrotyrosine (1:100 dil, 4°C overnight) followed by secondary antibody goat anti-rabbit biotinylated Ig (1:300 dil, room temp, 45 min) and then DAB chromogen. Sections were lightly counterstained with Myer's hematoxylin. To demonstrate specificity, negative controls included primary antibody preabsorbed with the antigen (nitrotyrosine).

Unfortunately, no evidence of capillary leakage was demonstrated in IL-2 + D-NAME group as compared to no treatment or IL-2 + L-NAME, indicating that the IL-2 therapy did not work as expected. This was likely due to a loss or diminution of activity of the IL-2 batch received 5 years ago from the Chiron Corporation. Nitrotyrosine staining patterns in the lungs of the three animal groups were also indistinguishable. Unexpectedly, all the lungs (including the normal controls) showed significant immunostaining for nitrotyrosine which was specific (see later).

Experiment 2

Since we could not obtain a fresh batch of IL-2 from the Chiron Corporation soon enough, our next experiment was to examine capillary leakage by increasing IL-2 doses to 50,000, 100,000 and 150,000 Cetus units/injection (n = 5/group). Significant leakage as indicated by a rise in wet/dry weight ratios of the lungs were only noted in the latter two groups, including some IL-2 induced morbidity, requiring sacrifice of a few animals before conclusion of all IL-2 doses. In addition to the lungs, kidneys were also subjected to immunostaining for nitrotyrosine. The results, presented in Figure 1 (lungs) and Figure 2 (Kidney), are summarized as follows. Lungs from both control and IL-2 treated mice showed significant specific immunostaining for nitrotyrosine in the alveoli and bronchioles, which was abolishied by preabsorption of the antibody with the excess antigen. The staining patterns were indistinguishable in the two groups (Figure 1). This is possibly because of significant production of NO as well as superoxide in the normal lungs leading to peroxynitrite and subsequently nitrotyrosine formation. Immunostaining patterns in the kidneys (Figure 2) revealed differences between the cortex and the medulla. Diffuse and patchy specific immunostaining of the cortex (glomeruli and convoluted tubules) was noted in both control and IL-2 treated groups, indicating the presence of nitrotyrosine. On the other hand, very little staining was seen in the renal medulla of control mice, whereas patches of specific immunostaining was noted in the collecting tubules and ducts of the renal medulla in IL-2 treated mice. Thus it appears that renal medulla provided some discrimination for nitrotyrosine as a marker for IL-2 induced capillary leakage.

We have just received a fresh batch of IL-2 from the Chiron Corporation. We shall repeat experiment 1 with IL-2 doses of 15,000 - 25,000 Cetus units/injection and focus our investigation on the kidneys to examine whether increased nitrotyrosine formation in IL-2 (+ D-NAME) treated mice can be mitigated with L-NAME. We shall also examine the cytotoxic effects of peroxynitrite on endothelial cells *in vitro*.

Legends to Figures 1 and 2, showing staining for nitrotyrosine

Figure 1 Sections of lungs: **(A)** and **(B)** from IL-2 (100,000 Cetus u/inj, every 8 h x 10 inj i.p.) treated mice; **(C)** and **(D)** from control (vehicle-treated mice). **(A)** and **(C)** show specific staining (brown) for nitrotyrosine in a variety of cells (epithial, endothelial and stromal cells) of the alveoli, which was abolished by preabsorption of the primary antibody with the antigen (nitrotyrosine) as shown in the sections from the corresponding lungs **(B** and **D)**. No difference in immunostaining pattern was detectable between the IL-2 treated and control mice.

Figure 2 Sections of kidneys: **A - D** from IL-2 (150,000 Cetus u/inj, every 8 h x 10 inj i.p.) treated mice and **E - H** from control (vehicle treated) mice. **A, B, E** and **F** represent sections of the cortex and **C, D, G** and **H** of the medulla. Pictures on the left **(A, C, E, G)** are sections treated with the primary antibody to nitrotyrosine and pictures on the right **(B, D, F, H)** are corresponding negative controls in which the primary antibody was preabsorbed with the antigen (nitrotyrosine). Specific immunostaining for nitrotyrosine is noted in cells of the cortex (glomeruli and convoluted tubules) both ini IL-2 treated **(A)** and control **(E)** mice. Patchy but strong immunostaining of collecting tubules and ducts in the medulla was noted in IL-2 treated **(C)** mice but not control vehicle treated **(G)** mice. Pre-absorption of the antibody with antigen abolished staining in all cases.

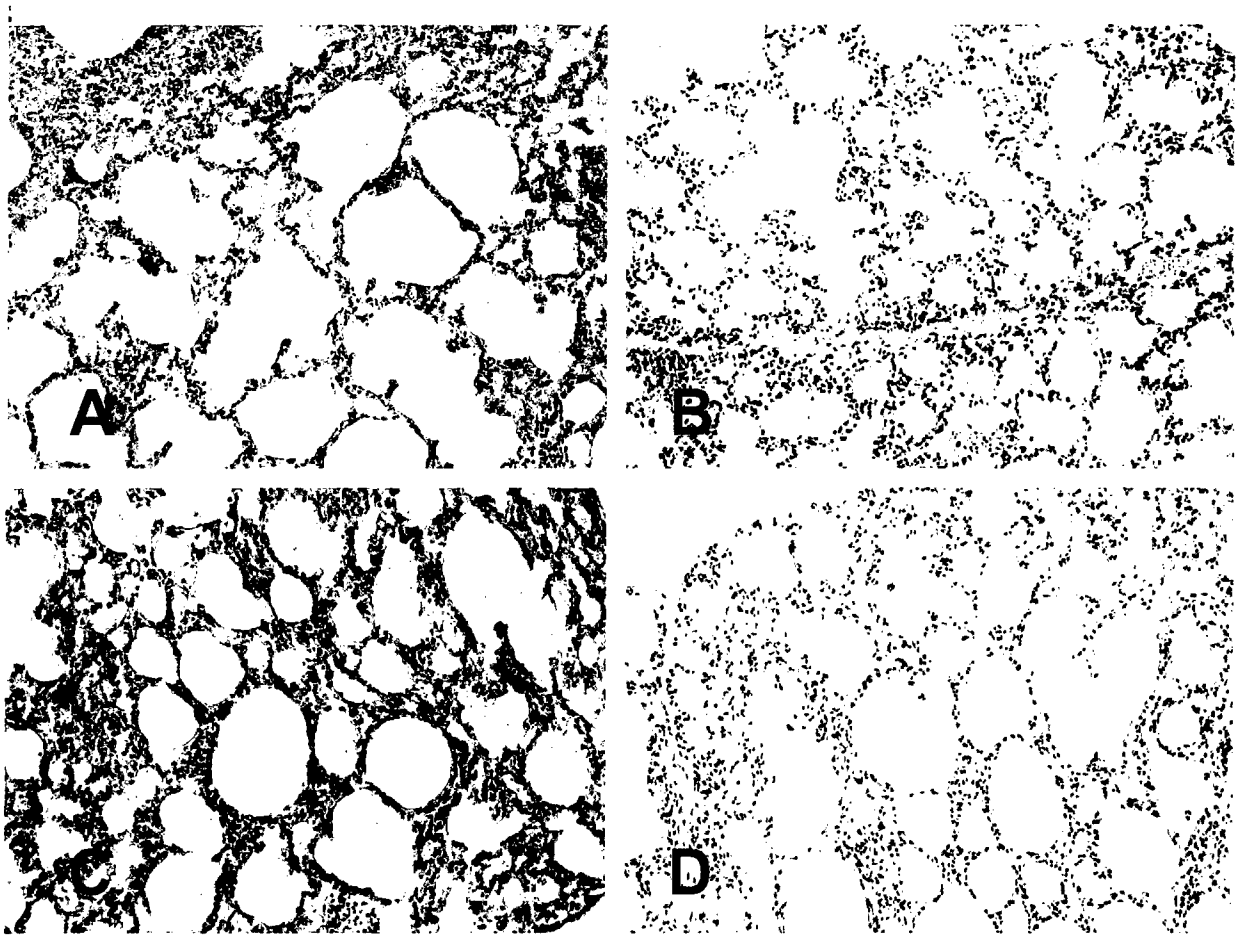

Figure 1

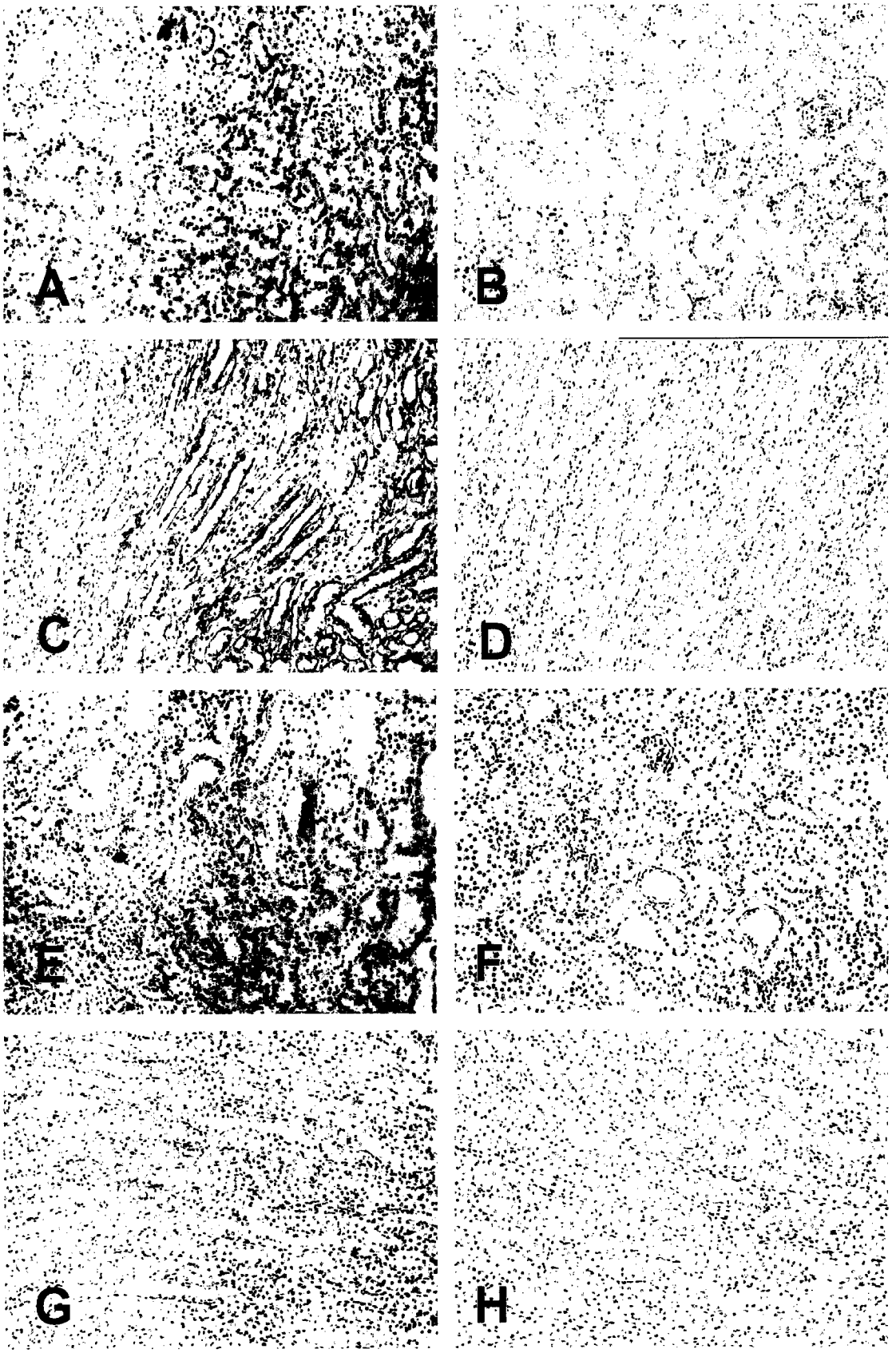

Figure 2

7. KEY RESEARCH ACCOMPLISHMENTS

Following were the achievements during the project period.

I. We have expanded and validated our earlier data showing that:

(a) Spontaneous primary C3H/HeJ tumors show a heterogeneity in eNOS-bearing tumor cells; this expression was unrelated to tumor growth rate. However, the incidence of eNOS bearing cells was higher in undifferentiated than in differentiated zones of primary tumors, and metastatic foci resulting from each primary tumor was mostly eNOS positive.

(b) All C3L5 tumor cells (a highly metastatic clone of a spontaneous tumor) expressed eNOS *in vitro*; a minority expressed iNOS under inductive conditions (IFN-γ + LPS). When transplanted *in vivo,* most tumor cells at the primary site and a high proportion at the metastatic site expressed eNOS. C10 tumor cells originally shown to be a poorly metastatic clone of the same spontaneous tumor were shown to have a lower *in vivo* growth rate of primary tumors and a lower rate of spontaneous lung metastasis than C3L5 cells. These differences were positively correlated with their differences in eNOS protein expression *in vitro* as well as *in vivo* in primary tumors but not in metastatic foci which were equally positive for eNOS.

These findings substantiated further our hypothesis that eNOS expression provided an advantage for metastasis.

II. We abandoned our futile attempts to knockout eNOS gene in C3L5 cells because we found that they have increased (3.6) number of gene copies. Subsequently we adopted the alternative approach of downregulating eNOS by antisense RNA transfection and isolated eNOS downregulated clones. However, all the clones proved to be unstable and thus could not be applied to functional assays *in vitro* or *in vito*. Currently, we are utilizing antisense oligos for short-term biological assays.

III. We have shown that endogenous NO promoted migratory function of C3L5 and C10 tumor cells.

This is the first definitive evidence of NO-mediated stimulation of tumor cell migration, which is an essential component of invasion and metastasis.

We shall examine the pathway for signal transduction for NO-mediated stimulation of tumor cell migration.

IV. **(a)** We have shown that endogenous NO promoted invasiveness of C3L5 and C10 tumor cells. The invasive function of the highly metastatic C3L5 cell line was investigated in detail. Their invasiveness was further stimulated by additional NO production when treated with IFN-γ and LPS because of the induction of iNOS in tumor cells.

This is the first definitive evidence of NO-mediated promotion of tumor cell invasiveness.

(b) We have identified some of the mechanisms responsible for NO-mediated stimulation of invasiveness. Endogenous and IFN-γ + LPS-induced NO down-regulated the expression of TIMP-2 and TIMP-3 genes. Induced NO further up-regulated the expression of MMP-2 gene. Thus, NO-mediated promotion of invasiveness resulted from an alteration in the balance between MMP-2 and TIMP's.

This is the first demonstration of mechanisms for NO-mediated promotion of tumor cell invasiveness.

IV. By devising a novel tumor-induced angiogenesis assay *in vivo,* we have obtained substantial data showing that endogenous NO promotes C3L5 tumor-induced angiogenesis, which was higher than the level of angiogenesis induced by C10 cells expressing a lower level of eNOS.

This novel and objective angiogenesis assay is highly suitable for testing anti-angiogenic agents against human tumor cells grown in nude mice.

We are currently testing the role of endogenous VEGF in eNOS activation in C3L5 cells.

V. We had shown that active, inducible NOS expression, leading to high NO production *in vivo* is responsible for IL-2 therapy-induced capillary leakage in healthy mice. We identified the iNOS-expressing cells in the vicinity of the leakage (pulmonary edema, pleural effusion) and have shown that NOS inhibitors can restrain the IL-2 therapy-induced structural damage to the lungs. We are conducting further studies to test our hypothesis that NO-mediated capillary damage following IL-2 therapy is owing to the formation of peroxynitrite.

In summary, our progress matched with our expectations in most areas. In one area we had slow progress. In other areas we had an accelerated progress, leading to some newer proposals for experimentation within the overall objectives of the project.

8. REPORTABLE OUTCOMES

A. Journal Publications (published, in press or submitted)

A1. Orucevic A, Hearn S, Lala PK: The role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome resulting from interleukin-2 therapy in mice. Lab Investigation. 76, 53-75, 1997.

A2. Lala PK, Orucevic A: Role of nitric oxide in tumor progression: Lessons from experimental tumors. Cancer and Metastasis Reviews. 17: 91-106, 1998.

A3. Orucevic A, Lala PK: Role of nitric oxide in interleukin-2 therapy induced capillary leak syndrome. Cancer and Metastasis Reviews. 17: 127-142, 1998.

A4. Orucevic A, Bechberger J, Green AM, Shapairo RA, Billiar TR and Lala PK: Nitric oxide production by murine mammary adenocarcinoma cells promotes tumor cell invasiveness. Int J Cancer 81: 889-896, 1999.

A5. Jadeski LC, Lala PK: NOS inhibition by N^G-Nitro-L-Arginine Methyl Ester (L-NAME) inhibits tumor-induced angiogenesis in mammary tumors. Amer J Path. In press, 1999.

A6. Jadeski LC, Hum KO, Chakraborty C, Lala PK: Nitric oxide promotes murine mammary tumor growth and metastasis by stimulating tumor cell migration, invasiveness and angiogenesis. Manuscript submitted for publication. 1999.

B. Conference presentations (Abstracts/extended abstracts)

B1. Lala PK, Hum K, Jadeski I, Orucevic A: Nitric Oxide (NO) mediated mammary tumor progression: Role of NO in tumor cell invasiveness. Proceedings of the Department of Defense Breast Cancer Program Meeting, Eara of Hope, Vol. 2, 709-710, 1997.

B2. Hum K, Lala PK: Nitric oxide synthase expression promotes murine mammary tumor progression and metastasis. Proc Amer Assoc Cancer Res 39: # 1450, 212, 1998.

B3. Jadeski L, Lala PK: Role of nitric oxide in mammary tumor angiogenesis. Proc Amer Cancer Res 39, # 2574, 378, 1998.

B4. Hum K, Jadeski L, Lala PK: Nitric oxide synthases and murine mammary tumor progression. Proc Amer Assoc Cancer Res 40: # 3715, 563, 1999.

B5. Lala PK, Hum K, Jadeski L: Nitric oxide (NO) synthase expression promotes growth and metastasis of murine breast cancer cells due to invasion and migration stimulation by NO. Abstract: Reasons for Hope Conference in Breast Cancer Research, sponsored by the Canadian Breast Cancer Research Initiative. p 185, 1999.

B6. Jadeski L, Lala PK: The role of nitric oxide in murine mammary tumor induced angiogenesis. Abstract: Reasons for Hope Conference in Breast Cancer Research, sponsored by the Canadian Breast Cancer Research Initiative. p 182, 1999.

9. CONCLUSIONS

Results of this project so far reveals that

(a) Tumor-derived nitric oxide promotes murine mammary tumor progression by multiple mechanisms including stimulation of tumor cell migration, invasiveness and tumor-induced angiogenesis.

Since NOS activity correlates with the progression of human breast cancer, the above information is highly relevant for designing breast cancer therapy in the human. NOS inhibitors should have a valuable role by blocking multiple steps in breast cancer progression and metastasis.

(b) Induction of iNOS leading to increased NO production in various tissue is responsible for IL-2 induced "capillary leak syndrome" which can be mitigated with NOS inhibitors. NOS inhibitors also improved the anti-cancer effects of IL-2 therapy.

High-dose IL-2 therapy, in spite of proven benefit in certain human cancers, has lately been abandoned because of this side effect. This therapy can now be reviewed in combination with selective iNOS inhibitors.

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APPENDICES

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NITRIC OXIDE PROMOTES MURINE MAMMARY TUMOUR GROWTH AND METASTASIS BY STIMULATING TUMOUR CELL MIGRATION, INVASIVENESS AND ANGIOGENESIS

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Running Title: NO stimulates migration, invasion and angiogenesis in mammary tumours
Grant sponsor: Department of the United States Army
Grant #: DMAD 17-96-6096 to PKL

Abstract

The present study examined the contributory role of NO on tumour growth and metastasis using a murine mammary tumour model which includes spontaneously-arising C3H/HeJ mammary tumours and two clonal derivatives with different metastatic phenotypes. Spontaneous tumours and their respective lung metastases were utilized to examine the relationship of NOS protein expression levels in tumour cells to the degree of morphological differentiation and growth rate of primary tumours and metastases. Two cell lines (C3L5, highly metastatic and C10, weakly metastatic), clonally derived from the same spontaneous tumour were utilized to investigate: a) the relationship between NOS protein expression, using *in vitro* and *in vivo* immunostaining, and the biological behavior of tumour cells by testing *in vitro* migratory and invasive capacities, or by examining *in vivo* tumour growth rate, and metastatic and angiogenic capacities, and (b) whether tumour-derived NO stimulated the invasive, migratory and angiogenic capacities of tumour cells.

Spontaneous primary tumours were heterogeneous in eNOS expression; irrespective of tumour growth rate, a mixture of strongly eNOS-positive and completely eNOS-negative cells was consistently observed. However, the expression was higher in undifferentiated relative to differentiated zones of tumour. Furthermore, most tumour cells in lung metastatic sites were strongly eNOS positive, suggesting a selective advantage of eNOS-expressing cells for growth and metastasis. This notion was supported by the findings using clonally-derived cell lines. Relative to C10 cells, C3L5 cells grew more rapidly at primary subcutaneous transplantation sites, metastasized to lungs more efficiently, and expressed higher levels of eNOS *in vitro* and *in vivo*. Nevertheless, lung metastases derived from both tumour cell lines were always strongly and homogeneously eNOS positive. Tumour cells did not express iNOS, but *in vivo* iNOS-expression was noted in some tumour-associated macrophages. For both cell lines, iNOS was equally inducible *in vitro* in the presence of LPS and IFN-γ. C3L5 cells were more invasive than C10 cells *in vitro*, but migratory capacities of the two cell lines did not differ. However, migration and invasiveness of both cell lines were inhibited in the presence of the NOS inhibitor L-NAME, with concurrent reduction in NO production (nitrate/nitrite); these functions were restored with excess L-arginine, indicating that the effects of L-NAME were NO-specific. Tumour-associated angiogenesis was higher in Matrigel implants containing C3L5 cells relative to those containing C10 cells. Furthermore, C3L5-induced angiogenesis was reduced *in vivo* with chronic L-NAME treatment, relative to D-NAME (inactive enantiomer) treatment. These findings suggest that tumour-derived NO, resulting from eNOS expression by tumour cells promoted tumour growth and metastasis in this mammary tumour model by multiple mechanisms: stimulation of tumour cell migration, invasiveness and angiogenesis.

Introduction

Nitric oxide (NO), an inorganic free radical gas, is synthesized from the amino acid L-arginine by a group of enzymes, the nitric oxide synthases (NOS). Three isoforms of the enzyme have been identified: endothelial (e) and neuronal (n) isoforms are Ca^{2+}/calmodulin-dependent, and are constitutively expressed. The inducible isoform (iNOS) is Ca^{2+}/calmodulin-independent, and induced in the presence of inflammatory cytokines or bacterial products. When constitutively expressed, NO produced at low levels is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation and neurotransmission. Under inductive conditions, high levels of NO can mediate antibacterial and antitumour functions, however, sustained, chronically-produced NO contributes to many pathological conditions including inflammation and cancer (reviewed by Moncada and Higgs, 1993; Knowles and Moncada, 1994).

The role of NO in tumour biology has been extensively studied; overall, an overwhelming majority of evidence suggests a positive association between NO and tumour progression. Overexpression of NOS enzymes and/or NOS activity were positively correlated with the degree of malignancy in human reproductive tract (i.e., ovarian, uterine) cancers (Thomsen et al., 1994), central nervous system tumours (Cobbs et al., 1995), and mammary tumours (Thomsen et al., 1995; Dueñas-Gonzalez et al., 1997). iNOS was detected in stromal elements, and eNOS was detected in tumour vasculature in a majority of gastric carcinomas (Thomsen and Miles, 1998), and, relative to benign prostatic hyperplasia, iNOS expression was higher in prostatic carcinomas (Klotz et al., 1998). Total NOS activity was shown to be increased in carcinomas of the larynx, oropharynx, oral cavity (Gallo et al., 1998), and adenocarcinomas of the lung (Fujimoto et al., 1997) relative to normal healthy control tissue.

Experimental tumour models have provided direct evidence of a promoting role of NO in tumour progression. Treatment with a NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) reduced NO production and tumour growth in a rat adenocarcinoma model (Kennovin et al., 1994). iNOS induction with lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine mammary tumour cells stimulated tumour growth and metastasis in vivo (Edwards et al., 1996). Furthermore, iNOS transduction in a human colon adenocarcinoma line resulted in enhanced tumour growth and vascularity when transplanted in nude mice (Jenkins et al., 1995). Our laboratory has studied the role of NO in tumour progression/metastasis using a murine mammary adenocarcinoma tumour model which includes spontaneously arising mammary tumours and their clonal derivatives. Preliminary studies of spontaneously-arising mammary tumours in C3H/HeJ retired breeder female mice revealed that tumour cells in primary tumours were distinctly heterogeneous in eNOS protein expression. However, a strong and homogeneous expression pattern was observed at metastatic lung sites in the same animals, suggesting that eNOS expression provided a selective advantage to metastasis (Lala and Orucevic, 1998). Further evidence supported this notion. A highly metastatic cell line, C3L5, clonally-derived from

a spontaneously-arising mammary tumour strongly expressed eNOS protein *in vitro* and *in vivo*, and iNOS upon stimulation with LPS and IFN-γ (Orucevic et al., 1999). Treatment of C3L5 mammary tumour-transplanted animals with the NOS inhibitors N^G-methyl-L-arginine (L-NMMA) or L-NAME significantly reduced primary tumour growth and lung metastases formation relative to control animals (Orucevic and Lala, 1996a; 1996b; Lala and Orucevic, 1998). The present study further examined the contributory role of NO in tumour progression/metastasis, and underlying mechanisms, in the murine mammary tumour model. We utilized spontaneous C3H/HeJ mammary tumours and two clonal derivatives of a spontaneous tumour with different capacities for spontaneous metastasis. A large number of spontaneous mammary tumours, and respective lung metastases, were utilized to examine the relationship between levels of NOS protein expression at primary tumour sites and the degree of morphological differentiation of tumour cells and tumour growth rates, and compare the levels of expression between the primary and metastatic lesions. Clonally-derived C3L5 (highly metastatic) and C10 (weakly metastatic) tumour cell lines were utilized to examine: a) the levels of NOS protein expression *in vitro* and *in vivo* at primary and metastatic tumour sites, and b) whether the levels of NOS protein expression or NO production by tumour cells were correlated with invasive or migratory abilities *in vitro* or to their biological behavior *in vivo* such as the rates of tumour growth and spontaneous lung metastases, and ability for angiogenesis, and c) whether NO production by tumour cells was causally related to invasion, migration and angiogenesis.

Materials and Methods

Mice

Female C3H/HeJ mice were obtained from Jackson Laboratory (Bar Harbor, Maine); retired breeder C3H/HeJ mice (approximately 6 months old) were studied in spontaneous mammary tumour experiments, and 6-8 week old C3H/HeJ mice were used in experiments related to tumour transplantation and angiogenesis with C3L5 and C10 cells. Upon arrival at the vivarium, animals were randomized into treatment groups; experimental procedures began after a one week acclimatization period. Throughout the investigation, animals had free access to food (standard mouse chow) and water, and were maintained on a 12 hour light/dark cycle. Animals were treated in accordance with guidelines set out by the Canadian Council on Animal Care

Spontaneous Mammary Tumour Development in C3H/HeJ Female Retired Breeder Mice

Fifty C3H/HeJ female retired breeder mice were monitored 2 times per week for spontaneous mammary tumour development (20 tumours studied) as previously reported (Lala et al., 1997). After initial localization of a primary tumour, tumour growth rate was monitored daily; minimum and maximum diameters were measured using digital calipers, and tumour volume calculated using the equation: tumour volume = $0.52a^2b$, where a and b were the minimum and maximum

tumour diameters, respectively (Baguley et al., 1989). When tumours had grown for 8 – 12 weeks, mice were sacrificed using an overdose of pentobarbital, primary tumours and lungs inclusive of metastatic foci were removed, processed for paraffin embedding, and immunostained for eNOS and iNOS antigens.

Tumour Cell Lines

Two murine mammary adenocarcinoma cell lines were utilized in the present research: C3L5, a highly metastatic line, and C10, a weakly metastatic line were both originally derived from a spontaneous tumour that developed in a C3H/HeJ female retired breeder mouse. Cells from the primary tumour (T58) demonstrated moderate metastatic capacity in early *in vitro* passages (Brodt et al., 1985). Two clones were then derived from T58 cells: the weakly metastatic C10 line currently used, and C3, a highly metastatic line. Since the metastatic capacity of the C3 line declined after several years of repeated *in vitro* passages (Lala et al., 1986), a highly metastatic C3L5 line was derived by five cycles of repeated *in vivo* selections for spontaneous lung micrometastases following subcutaneous transplantation of C3 cells into C3H/HeJ mice and retransplantation of dispersed lung micrometastases subcutaneously in syngeneic mice. This led to the production of the highly metastatic cell line C3L5 (Lala and Parhar, 1993) which has since maintained its strong metastatic phenotype. The C3L5 and C10 cells used in the present research were grown from frozen stock and maintained in RPMI 1640 medium (GIBCO; Burlington, ON) supplemented with 5% fetal calf serum (GIBCO; Burlington, ON) and 1% penicillin-streptomycin (Mediatech; Washington, DC) in a humidified incubator, 5% CO_2.

Tumour Transplantation Studies

C3L5 and C10 cells, grown in monolayer, were harvested by brief exposure to 0.05% trypsin-PBS-EDTA solution. Five X 10^5 C3L5 or C10 cells, suspended in 0.5 ml of RPMI, were injected subcutaneously in the mammary line in the left axillary region of 6 to 8 week old C3H/HeJ female mice (n = 15 mice per group). Trypan blue exclusion staining ensured adequate tumour cell viability (i.e., > 95%) Tumour growth was monitored using digital calipers; 2 times per week, the minimum and maximum diameters were recorded, and tumor volume calculated as described for spontaneous tumours. Twenty-one days after tumour transplantation, mice were sacrificed using an overdose of pentobarbital, and primary tumours removed, fixed in 4% paraformaldehyde, and processed for paraffin embedding. Lungs were inflated *in situ* with Bouin's fixative, removed and assessed for lung surface colonies using a dissecting microscope (experimenter blind to experimental condition). Immunostaining of both primary tumours and the corresponding lung metastases for eNOS and iNOS protein was conducted as described below.

Immunocytochemical Detection of NOS Enzymes in Cells Propagated In Vitro

C3L5 and C10 cells were grown in complete RPMI 1640 medium alone, or in complete medium containing IFN-γ (1000 U/ml; Gibco BRL, Grand Island, NY) and LPS (100 ng/ml); Sigma Chemical Co., St. Louis, MO) for 24 hours on chamber slides (Nunc, Naperville, IL) in a humidified incubator (37°C, 5% CO_2). Cells were fixed in ice-cold methanol (-20°C, 5 minutes). Endogenous peroxidase activity was blocked with methanol containing 3% H_2O_2 (room temperature, 5 minutes), and cell membranes permeabilized using 0.25% Triton X-100 in 0.2% bovine serum albumin (BSA: Boerhinger Mannheim, Penzberg, Germany) in PBS, prior to application of blocking antibody: normal horse serum diluted in 0.2% BSA (1:10; 1 hour at room temperature in humidified chamber). Cells were then incubated with primary antibody: mouse monoclonal anti-eNOS or mouse monoclonal anti-macrophage iNOS (1:80 diluted in 0.2% BSA; overnight at 4°C, or 1:50 diluted in 0.2% BSA; overnight at 4°C; Transduction Laboratories, Lexington, KY) for eNOS and iNOS localization, respectively. Secondary antibody: biotinylated horse anti-mouse (1:200 diluted in 0.2% BSA; 1 hour at room temperature) was then applied, followed by avidin-biotin complex (ABC; Vector Laboratories, Inc., Burlingame, CA) (1 hour at room temperature) and DAB chromogen (Sigma Chemical Company, St. Louis, MO). Negative controls were incubated with the equivalent concentration of mouse IgG (DAKO, Horsholm, Denmark) in place of primary antibody. Immunocytochemical staining of human umbilical vein endothelial cells (HUVEC) for eNOS served as positive controls.

Immunohistochemical Detection of NOS Enzymes in Spontaneous and Transplanted Tumours

Paraformaldehyde fixed, paraffin embedded primary tumours and lungs inclusive of metastatic foci were sectioned at 7 μm thickness. Following deparaffinization and rehydration of sections, endogenous peroxidase activity was blocked using methanol containing 3% H_2O_2 prior to application of blocking serum, and primary and secondary antibodies, as described above. Sections were lightly counterstained with Mayer's Haemalum, and those sections used to quantify immunostaining intensities were incubated with metal enhanced DAB (Pierce, Rockford, IL), and not counterstained.

Quantitative Analysis of Immunohistochemically Stained Tissue Sections

The intensity of immunohistochemical staining was quantified using a method similar to those described by Lehr et al. (1997), Childs and Unabia (1997) and Wang et al. (1997). Digitized images of non-counterstained primary tumour sections and lung metastases were obtained, and imported into the image analysis software program Mocha (Jandel Scientific, San Rafael, CA); pixels of the black and white images were inverted and remapped (i.e., black pixels converted to white, and white converted to black), therefore absolute white was measured as 0 and absolute black as 255 grey level units. The average intensity of immunohistochemical staining in healthy

(non-necrotic) tumour tissue was quantified and compared between experimental groups (i.e., C3L5 and C10) and corresponding negative control sections.

In Vitro Invasion and Migration Assays

Both invasion and migration assays were conducted in transwells fitted with millipore membranes (6.5 mm filters, 8 μm pore size; Costar Corp., Toronto, ON). In the invasion assay, cells degraded and passed through a Matrigel barrier prior to migrating through membrane pores. Thus, in invasion assay, membranes were coated with 120 μl growth factor-reduced Matrigel (1:20 dilution in RPMI 1640 medium; Collaborative Research, Bedford, MA). For both assays, 2.5 X 10^4 C3L5 or C10 cells/100 μl complete RPMI were plated in upper wells of transwell chambers containing either 200 μl complete RPMI, complete RPMI and L-NAME (0.01, 0.1, or 1 mM); Sigma Chemical Co., St. Louis, MO), or complete RPMI, L-NAME and excess L-arginine (5 mM; Sigma Chemical Co., St. Louis, MO) (total volume in upper chamber; 200 μl). Bottom wells contained 800 μl complete RPMI. Chambers were gently shaken for 1 hour at room temperature, followed by 24, 48 or 72 hour incubation (37°C, 5% CO_2). After incubation, cells from the upper surface of millipore membranes were completely removed with gentle swabbing, remaining migrant cells were fixed and stained using Diff-Quik® Stain Set (Dada AG, Dudingen, Switzerland). Membranes were then rinsed with distilled H_20, gently cut from transwells and mounted onto glass slides with Aquapolymount. Cellular invasion and migration indices were determined by counting the number of stained cells on membrane in 5 randomly selected, non-overlapping fields at 400X magnification under a light microscope (researcher blind to experimental condition).

Assay for In Vitro NO Production

C3L5 and C10 cell culture media was collected at the same time points, and under identical conditions used in invasion and migration assays, and stored at –20°C until assayed. Levels of NO were measured by determining levels of inorganic NO_2–, a stable product of oxidized NO (Moncada and Higgs, 1993) in the Greiss reaction (Green et al., 1982) using a procedure previously established in this laboratory (Orucevic and Lala, 1996a). Briefly, samples of culture medium were diluted in deionized H_20 (1:1), and proteins precipitated using 50 μl 30% $ZnSO_4$ and 1 ml of dilute sample, followed by centrifugation (8000g, 5 minutes); 1 ml supernatant was incubated (room temperature, 30 minutes) with 300 μl 0.5M ammonium chloride, 100 μl 0.06M sodium borate and 50 mg acid-washed cadmium filings (Davison and Woof, 1978). The mixture was centrifuged (400g, 7 minutes), and 1 ml supernatant added to Greiss reagent and incubated (room temperature, 10 minutes). Greiss reagent was prepared by mixing equal parts 1% sulphanilic acid (Sigma Chemical Co., St. Louis, MO) and 0.1% naphthylethylenediamine (Sigma Chemical Co., St. Louis, MO) in 2% phosphoric acid. Absorbance of samples at 543 nm was

measured using spectrophotometer; concentration of NO_2- in media samples was determined from sodium nitrite standard curve, which was linear for 0 to 100 μM of nitrite.

In Vivo Tumour-induced Angiogenesis Assay

Levels of tumour-induced angiogenesis *in vivo* were quantified using a novel assay devised in our laboratory, and based on our observation that angiogenesis was induced in implants of growth factor-reduced Matrigel, only when tumour cells were suspended in the Matrigel (Jadeski and Lala, 1999). We compared angiogenesis induced by C3L5 and C10 cells, and examined the effects NOS inhibition by chronically administering L-NAME, or its inactive enantiomer, D-NAME to mice using osmotic minipumps.

In the inguinal region, mice received subcutaneous implants of 5×10^4 C3L5 or C10 cells suspended in Growth Factor Reduced Matrigel® (Collaborative Research, Bedford, MA) (3.5 mg Matrigel in 0.5 ml RPMI), and on the contralateral side as controls, the equivalent amount of Matrigel alone. Immediately thereafter, osmotic minipumps (©ALZA Corporation, Palo Alto, CA) were implanted subcutaneously, providing a constant systemic supply (0.5 ml per hour; 25 mg per 200 μl 0.9% NaCl) of L-NAME (n = 15 per group), or D-NAME (n = 15 per group) (both drugs purchased from Sigma Chemical Co., St. Louis, MO) for the experiment duration (14 days).

Mice were sacrificed using an overdose of pentobarbital, and Matrigel implants removed, fixed in 4% paraformaldehyde, processed for paraffin embedding, sectioned and stained with Masson's Trichrome. Sections were scanned at low power for areas containing new blood vessels (researcher blind to experimental condition); these areas were systematically imaged at 160X magnification using Northern Exposure (Empix Imaging Inc.), and individual vessel counts for each field were documented using Mocha™ Image Analysis Software (Jandel Scientific) to identify fields of maximum blood vessel density (i.e., 'hot spots'). Subsequently, 'hot spots' were statistically analyzed for between-group differences by determining the average (n = 15 animals per group) of the average of three fields of maximal blood vessel density (taken in descending order) per animal.

Statistical Analysis

Data were analyzed using the SAS system for windows—release 6.12. Data comparing two means were tested using Student's T-tests; those comparing multiple (i.e., more than 2) means, for a single main effect, were tested using one-way analysis of variance (ANOVAs). Data from the *in vivo* pulmonary metastasis assay were analyzed using the Mann-Whitney Rank Sum Test. Main effects of tumour type (i.e., C3L5 and C10) and treatment (i.e., L-NAME and D-NAME) were tested for the dependent variable (blood vessel formation) using two-way ANOVAs in the *in vivo* angiogenesis assay. A probability of 0.05 was always used in determining statistical significance.

RESULTS

Primary Tumour Growth Rate and Metastatic Lung Colony Formation in C3H/HeJ Mice after Subcutaneous Injection of 5 X 10^5 C3L5 or C10 Cells

Figure 1 shows volumes of primary tumours and number of lung metastases forming in C3H/HeJ mice receiving subcutaneous implants of C3L5 or C10 tumour cell lines (n = 15 mice per group). C3L5-derived primary tumours grew faster, resulting in larger tumours, than those derived from C10 cells at day 14 ($P < 0.0001$) and day 21 ($P < 0.0001$) after tumour transplantation (Figure 1a). In addition, the number of spontaneous metastatic lung colonies harvested at 21 days was higher in mice receiving subcutaneous implants of C3L5 cells relative to those receiving C10 cells (C3L5, 199.6 ± 25.0; C10, 54.1 ± 18.3, $P < 0.001$).

Immunocytochemical Detection of NOS (eNOS and iNOS) Enzymes In Vitro and In Vivo

(a) In Vitro Propagated Cells:

Strong and homogeneous eNOS staining was observed *in vitro* in 100% of cultured C3L5 mammary adenocarcinoma cells, as reported earlier (Orucevic And Lala, 1998), suggesting that these cells constitutively express high levels of eNOS. In contrast, *in vitro* eNOS immunostaining in C10 cells was much weaker and more heterogeneous relative to C3L5 cells (data not shown). Under normal culture conditions, C3L5 and C10 cells did not express iNOS, however, iNOS expression was induced in both cell lines using IFN-γ and LPS; both cell lines exhibited moderate iNOS staining in approximately 40-50% of cells (data not shown).

(b) Spontaneous Mammary Tumours and Their Metastases:

Spontaneous mammary tumours developing in C3H/HeJ female retired breeder mice (tumour size at 8-12 weeks tumour age: 8-20 mm diameter) showed localized variations in levels of morphological differentiation within primary tumours, irrespective of tumour growth rate. Differentiated zones were comprised of tumour cells arranged in pseudoacini, whereas poorly differentiated sites constituted spindle shaped tumour cells arranged in sheets, whorls or clusters. A heterogeneous pattern of eNOS expression was seen in both differentiated and poorly differentiated zones of tumour tissue; cells were either strongly eNOS positive or completely eNOS negative (differentiated zone: Figure 2A), however, the proportion of eNOS-positive cells was consistently higher in poorly differentiated zones relative to differentiated zones (data not shown). Overall, approximately 40-70% of tumour cells in individual primary tumours were eNOS positive. A clear correlation between eNOS expression patterns and tumour growth rates was not observed (high tumour growth rate: 13-20 mm tumour diameter at 8-12 weeks, n = 4; moderate growth rate: 8-12 mm, n = 11; low growth rate: < 8 mm, n = 5). In contrast, all spontaneous lung metastases were strongly and homogeneously eNOS positive; virtually all (76-100%) tumour

cells at metastatic sites expressed eNOS (Figure 2C). Tumour cells within primary and metastatic sites did not express iNOS, however, some tumour-associated macrophages, located in the primary tumour tissue and surrounding stroma (Figure 2D), and occasionally at metastatic sites (data not shown), stained positively for iNOS.

(c) Transplanted C3L5 and C10 Primary Tumours and Their Spontaneous Lung Metastases:

Figures 2E-H show eNOS staining in primary and metastatic tumours 3 weeks after transplantation of C3L5 (Figure 2E, primary tumour; Figure 2F, lung metastasis) and C10 (Figure 2G, primary tumour; Figure 2H, lung metastasis) cells. Primary tumours derived from the weakly metastatic C10 cell line showed weak and heterogeneous eNOS staining; relatively weak eNOS positivity was noted in approximately 20-50% of tumour cells (Figure 2G). Primary tumours derived from the highly metastatic C3L5 cell line (Figure 2E) were strongly and homogeneously eNOS positive; most (>90%) tumour cells expressed eNOS. However, regardless of tumour cell line transplanted (i.e., C3L5 or C10), lung metastases were always strongly and homogeneously eNOS positive; approximately 60-80% of tumour cells within metastatic lung colonies showed strong staining for eNOS (Figure 2F, C3L5; Figure 2H, C10). Tumour cells within primary tumours and metastases did not express iNOS. However, iNOS expression was observed in tumour-associated macrophages at primary and metastatic sites of both tumour types (data not shown).

Quantification of NOS Staining Intensity of Primary Tumours and Lung Metastases in Mice Bearing C3L5 or C10 Tumour Transplants, Using Computer Assisted Image Analysis

Figure 3 shows quantification of the staining intensity within primary, healthy (non-necrotic) tumour tissue and lung metastases (Figures 3A and 3B, respectively) harvested 3 weeks after transplantation of C3L5 or C10 cells. The average intensity of immunohistochemical staining for eNOS was higher in tumour cells within primary tumours derived from C3L5 cells relative to those derived from C10 cells (C3L5, 52.879 ± 4.48; C10, 7.543 ± 1.27, $P < 0.001$). Since eNOS staining intensity did not differ in regions of tumour sections containing blood vessels versus those devoid of blood vessels, and this relationship was observed for both C3L5 and C10-derived tumours (C3L5: blood vessel-containing regions, 53.163 ± 4.85; blood vessel-devoid regions, 54.034 ± 4.546, $P = 0.8969$) (C10: blood vessel-containing regions, 4.543 ± 1.270; blood vessel-devoid regions, 7.326 ± 1.162, $P = 0.9012$), it appears that endothelial cells did not alter the relative amount of eNOS staining between C3L5 and C10-derived tumours. The intensity of eNOS immunostaining did not differ in lung metastases derived from C3L5 and C10 cells (C3L5, 53.461 ± 2.647; C10, 52.644 ± 1.795, $P = 0.8008$). iNOS immunostaining was higher in metastatic lung colonies derived from C3L5 relative to those derived from C10 cells (C3L5, 17.938 ± 0.386; C10, 15.918 ± 0.704, $P > 0.0197$).

Kinetics of In Vitro Migration and Invasion by C3L5 and C10 Cells

The temporal kinetics of migration and invasion are shown in Figures 4A and 4B, respectively. Migration rates of the two cell types did not differ ($P = 0.429$ at 72 hours), however, C3L5 cells were more invasive than C10 cells (C3L5: 86.20 ± 3.60; C10: 57.8 ± 6.92, $P < 0.0011$ at 72 hours).

Migration and invasiveness of C3L5 and C10 cells under different treatment conditions

The effects of L-NAME treatment at various doses ± 5-fold excess L-arginine (natural substrate for NOS; competes with L-NAME and blocks NO-specific effects of L-NAME) were examined on the migratory and invasive abilities of both cell lines at 24, 48 and 72 hours. Since the effects were qualitatively similar at all time points, only the 72 hour time point is shown for migration and invasion (Figures 5A and 5B, respectively). L-NAME treatment at varying doses (i.e., 0.01 mM, 0.1 mM and 1 mM L-NAME) reduced the migratory capacity of both C3L5 ($P < 0.0001$) and C10 ($P < 0.0001$) cells relative to untreated control cells. Migratory capacities of both cell lines were restored to baseline levels after additional treatment with excess L-arginine, indicating that the inhibitory effects of L-NAME on migration were NO-specific.

The invasion indices of C3L5 and C10 cells after 72 hours incubation, under different treatment conditions, is shown in Figure 5B. As shown earlier, C3L5 cells invaded matrigel barrier at a faster rate relative to C10 cells ($P < 0.0011$). In addition, L-NAME treatment at varying doses (i.e., 0.01 mM, 0.1 mM and 1.0 mM L-NAME) reduced the invasive capacity both of C3L5 ($P < 0.0001$) and C10 ($P < 0.0001$) cells relative to untreated control cells. Invasive capacities of both cell lines were restored to near baseline levels after additional treatment with excess L-arginine, indicating that the effects of L-NAME on invasion were NO-specific.

NO Production Assay

Figure 6 shows the levels of NO produced by C3L5 and C10 cells as measured by the nitrate/nitrite levels present in the culture media collected after 72 hours incubation, under the treatment conditions examined in invasion and migration assays. Under normal culture conditions, nitrate/nitrite levels were higher in culture media from C3L5 cells relative to that from C10 cells (C3L5, 78.405 ± 1.193; C10, 67.425 ± 0.506, $P < 0.0001$). For both cell lines, treatment of cells with L-NAME at various doses (i.e., 0.01 mM, 0.1 mM and 1 mM) reduced levels of nitrate/nitrite produced relative to untreated control cells (C3L5, $P < 0.0001$; C10, $P < 0.0001$), and these levels were restored with the additional treatment with excess L-arginine.

In Vivo Tumour-induced Angiogenesis Assay

Figure 7 shows the number of blood vessels per unit area in Matrigel implants containing C3L5 or C10 cells, retrieved from animals treated with L-NAME or D-NAME (control animals). C3L5 cells were more angiogenic than C10 cells; in control animals treated with D-NAME, neovascularization was lower in C10-containing implants relative to those containing C3L5 cells (C3L5, 71.87 ± 5.33; C10, 39.075 ± 4.868, $P < 0.0001$). L-NAME therapy reduced angiogenesis in C3L5-containing implants relative to control animals, but did not affect neovascularization in C10-containing implants (C3L5: L-NAME, 34.177 ± 4.68 D-NAME, 71.87 ± 5.33, $P < 0.0001$) (C10: L-NAME, 38.14 ± 4.68 D-NAME, 39.08 ± 4.87, $P = 0.8903$).

DISCUSSION

The present study investigated the role of endogenous NO, resulting from eNOS expression by tumour cells, in tumour progression and metastasis using the C3H/HeJ murine mammary tumour model which includes spontaneously arising tumours and two clonal derivatives differing in their metastatic phenotype. An examination of a large number of spontaneous tumours confirmed our preliminary findings (Lala and Orucevic, 1998) that tumour cells at primary sites were distinctly heterogeneous in eNOS protein expression, whereas those at spontaneous lung metastatic sites had a strong and homogeneous expression pattern, suggestive of a metastasis-promoting role of eNOS. This concept was further validated by our findings of a positive correlation between the levels of eNOS expression in primary tumours and the primary tumour growth rate or formation of spontaneous pulmonary metastases of two transplanted, clonally derived cell lines: C10, a weakly metastatic cell line (Lala et al., 1986), and C3L5, a highly metastatic cell line (Lala and Parhar, 1993).

In spite of differences in eNOS expression *in vitro,* and *in* vivo, at primary transplant sites, eNOS expression was equivalent in tumour cells at metastatic sites for both tumour lines, as revealed with objective, computer-assisted analysis. Exclusion of eNOS positive vascular endothelial cells *in vivo* did not alter these results. Expression of iNOS was observed in a subset of macrophages located within the primary tumour tissue, the surrounding stroma, and metastatic sites of both spontaneous and transplanted tumours. iNOS expression did not differ between C3L5 and C10-derived tumours, therefore it is likely that the observed differences in tumour growth and metastasis were eNOS mediated. However, this relationship, on its own, did not establish causality. A causal relationship between NO production and tumour progression was earlier demonstrated in C3L5 tumour-bearing mice; antitumour and antimetastatic effects were observed with NOS inhibition using NMMA and L-NAME (Orucevic and Lala, 1996a, 1996b). The present study further explored the underlying mechanisms utilizing both tumour cell lines.

Key cellular processes that dictate primary tumour growth rate are tumour cell proliferation, survival and ability for angiogenesis. Invasiveness and metastasis depend on migratory as well as matrix-degrading abilities of tumour cells. These processes can be quantified with appropriate *in vitro* and *in vivo* assays. We earlier found that endogenous NO did not alter C3L5 cell proliferation (Orucevic et al., 1999); we have also observed that *in vitro* growth rates of C10 and C3L5 cells did not differ (unpublished). In the present study we compared migratory, invasive and angiogenic abilities of the two cell lines and the role of endogenous NO in each of these events.

In spite of differences in eNOS protein expression and NO production by the two tumour cell lines in vitro, there was no significant difference in the migratory abilities of C10 and C3L5 cells. However, migration, and NO production by both cell lines were inhibited in the presence of L-NAME, and the inhibitory effects were abrogated in the additive presence of L-arginine, indicating that endogenous NO had migration stimulating effects in both cases. Thus, the observed absence of differences in the basal migration rates of the two cell lines can only be explained by the presence of additional migration regulatory factor(s) differentially expressed by the two cell lines. Present report, to our knowledge, is the first demonstration of migration-promoting role of endogenous NO in tumour cells. The precise pathway of signal transduction responsible for this role remains to be examined.

C3L5 cells were more invasive than C10 cells, consistent with the differences in their invasive and metastatic abilities in vivo. Invasiveness as well as NO production by both cell lines was suppressed in the presence of L-NAME and restored with the additional presence of L-Arginine, showing that endogenous NO had invasion-promoting effects for both cells, and that differences in their invasive abilities could be explained, at least in part, on the basis of their differences in NO-producing abilities. Cellular invasiveness depends on multiple steps including matrix-degrading and migrating abilities. Since the latter function was indistinguishable in the two cell lines, it is likely that the differences in their invasiveness were due to differences in their matrix-degrading capabilities. Indeed, earlier work from this laboratory has demonstrated that endogenous NO promotes matrix degradation by C3L5 cells by a differential regulation of matrix degrading enzymes and their natural inhibitors. Constitutive NO production by C3L5 cells was shown to down regulate TIMP-2 and TIMP-3 mRNA, whereas additional NO production induced in the presence of LPS and IFN-γ, also caused an upregulation of MMP-2 mRNA (Orucevic et al., 1999). Additional mechanisms responsible for NO-mediated stimulation of cellular invasiveness may exist. For instance, NO has been shown to stimulate degradation of articular cartilage by stimulating other MMPs (collagenases and stromelysin) in human, bovine and rabbit chondrocytes (Murrell et al., 1995; Tamura et al., 1996).

Finally, the present study revealed that C3L5 and C10 cells differed in their angiogenic abilities in vivo, consistent with the differences in their NO-producing abilities. This may explain,

at least in part, the observed differences in the growth rates of the primary tumours tumours following transplantation of equivalent number of C3L5 and C10 cells at identical sites in syngeneic mice. That endogenous NO promoted angiogenesis was shown in the case of C3L5 cells by a significant reduction of angiogenesis in L-NAME-treated in comparison with D-NAME-treated mice. This finding is a confirmation of our earlier studies with this cell line using this novel angiogenesis assay devised in our laboratory (Jadeski and Lala, 1999). However, no significant reduction of angiogenesis was observed in matrigel implants of C10 cells in mice subjected to L-NAME therapy. This can be explained by two possibilities: 1) a differential expression of other angiogenesis-regulating factor(s), and 2) a requirement of a certain threshold level of NO production for stimulation of angiogenesis in the present tumour model. Our findings that the levels of angiogenesis were similar in L-NAME-treated C3L5 and D-NAME or L-NAME-treated C10 implants support the second but do not exclude the former possibility. Angiogenesis-promotion by NO has been reported in other conditions utilizing *in vitro* and *in vivo* assays (Ziche et al., 1993, 1994, 1997). This has also been demonstrated in tumour models utilizing other approaches. For example, iNOS overexpression in a human colonic adenocarcinoma cell line led to increased growth rate of cells in vivo in nude mice, in association with increased vascularity (Jenkins et al., 1995). Furthermore, NOS inhibition reduced angiogenesis in the rabbit cornea following xenotransplantation of human squamous cell carcinoma cells (Gallo et al., 1998). Taken together, these findings suggest that promotion of angiogenesis is a key event responsible for NO-mediated stimulation of tumour growth and metastasis.

In summary, present studies in the C3H/HeJ murine mammary tumour model have established that tumour-derived NO played a stimulatory role in tumour progression and metastasis by multiple mechanisms: promotion of migration, matrix degradation and angiogenesis. Thus, NOS inhibitors may prove to be important components of combination therapy protocols in certain human tumours, including breast cancer which exhibits a positive association of NOS activity with tumour grade (Thomsen et al., 1995; Dueñas-Gonzalez et al., 1997).

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Figure Legends

Figure 1

Primary tumour growth rate and metastatic lung colony formation in C3H/HeJ mice after subcutaneous injection of 5 X 10^5 C3L5 or C10 tumour cells.

Figure 1A: At 14 and 21 days after tumour transplantation, the primary tumour volumes were significantly lower (*; $P < 0.0001$) for C10 relative to C3L5 transplants. Data represent mean ± S.E.; n = 15 per group. Figure 1B: Mean number of metastatic lung nodules 3 weeks after tumour transplantation. Fewer lung colonies formed in mice bearing C10 relative to C3L5 tumour transplants (*; $P < 0.001$). Data represent mean ± S.E.; n = 15 per group.

Figure 2

Immunohistochemical localization of eNOS and iNOS proteins in spontaneous (Figures 2A-2D) and transplanted (Figures 2E-2G) mammary tumours (C3L5 or C10) at primary and metastatic sites.

A: Spontaneous primary tumour showing both eNOS positive and negative tumour cells arranged in pseudoacinar formation. B: Representative negative control. C: Lung metastasis of tumour depicted in Figure 2A; metastases were always strongly and homogeneously eNOS positive. D: Spontaneous primary tumour immunostained for iNOS; macrophages, present in tumour stroma, stained positively for iNOS, whereas tumour cells did not express iNOS.

E: Subcutaneously transplanted C3L5 primary tumours exhibited strong eNOS positivity relative to C10-derived tumours (G). Constitutive eNOS protein was expressed in endothelial cells lining blood vessels in tumour tissue. eNOS expression was similar in metastatic lung colonies arising after C3L5 or C10 transplantation (F and H, respectively).

Figure 3

Quantification of the average staining intensity of primary tumours from C3H/HeJ mice bearing C3L5 or C10 tumour transplants immunostained for eNOS protein.

Figure 3A: The intensity of immunohistochemical staining for eNOS was higher in tumour cells within primary C3L5 tumours relative to those derived from C10 cells ($P < 0.001$). eNOS immunostaining intensity did not differ in regions containing blood vessels versus those devoid of blood vessels, and this was observed for both C3L5 and C10-derived tumours (C3L5, $P = 0.8969$; C10, $P = 0.9012$), indicating that endothelial cells did not alter the relative amount of eNOS staining between C3L5 and C10-derived tumours. Figure 3B: The intensity of eNOS immunostaining did not differ in lung metastases derived from C3L5 and C10 cells ($P = 0.8008$). iNOS immunostaining was higher in lung metastases derived from C3L5 cells relative to those derived from C10 cells ($P < 0.0197$).

Figure 4

Temporal kinetics of in vitro migration and invasion

Figure 4A: Kinetics of migration of C3L5 and C10 tumour cells; C3L5 and C10 cells did not differ in their migratory capacity (n = 20 fields per group). Figure 4B: Kinetics of invasion of C3L5 tumour cells; invasion rate of C10 cells was slower relative to C3L5 cells ($P < 0.0011$; n = 20 fields per group).

Figure 5

Migration and invasion indices of C3L5 and C10 cells under different treatment conditions (72 hour incubation).

Figure 5A: Migratory ability of both C3L5 and C10 cells was reduced after treatment with L-NAME at various concentrations (0.01, 0.1 and 1 mM) relative to control cells (C3L5, $P < 0.0001$; C10, $P < 0.0001$), and migration of both cell lines was restored to basal levels after additional treatment with L-arginine (0.01, 1.0 mM). Figure 5B: Treatment of C3L5 and C10 cells with L-NAME reduced their invasive abilities relative to untreated controls (C3L5, $P < .0.0001$; C10, $P < 0.0001$) Additional treatment with L-arginine increased invasion of both cell lines, restoring it to near basal levels.

Figure 6

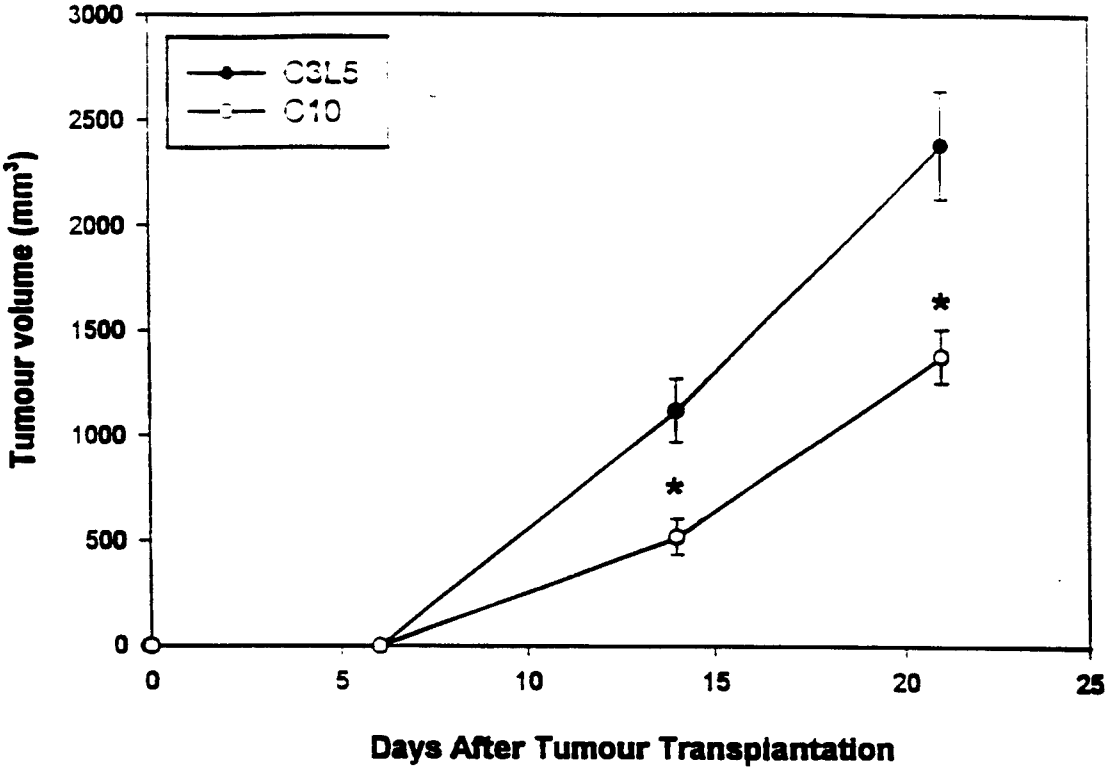
NO production by C3L5 and C10 cells measured by nitrate/nitrite levels in culture media under different treatment conditions (72 hour incubation).

Concentrations of nitrate/nitrite were higher for C3L5 relative to C10 cells ($P < 0.0001$). Relative to untreated C3L5 and C10 cells, production of NO by both cell lines decreased significantly after treatment with L-NAME at various concentrations (0.01, 0.1, 1.0 mM) (C3L5, $P < 0.0001$; C10, $P < 0.0001$). Levels of nitrate/nitrite increased, and were restored to near basal levels with additional exposure of C3L5 and C10 cells to L-arginine (0.01, 1.0 mM).

Figure 7

Levels of angiogenesis in C10 and C3L5 implants in L-NAME-treated and control (D-NAME-treated) animals.

For control animals treated with D-NAME, the neovascular response was higher in Matrigel implants containing C3L5 cells relative to those containing C10 cells ($P < 0.0001$). L-NAME-treatment reduced angiogenesis in implants containing C3L5 cells relative to control animals ($P < 0.0001$), but did not alter angiogenesis in implants containing C10 cells relative to control animals ($P = 0.8903$).


C3L5
C10
Tumour volume (mm³)
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Days After Tumour Transplantation
*
*

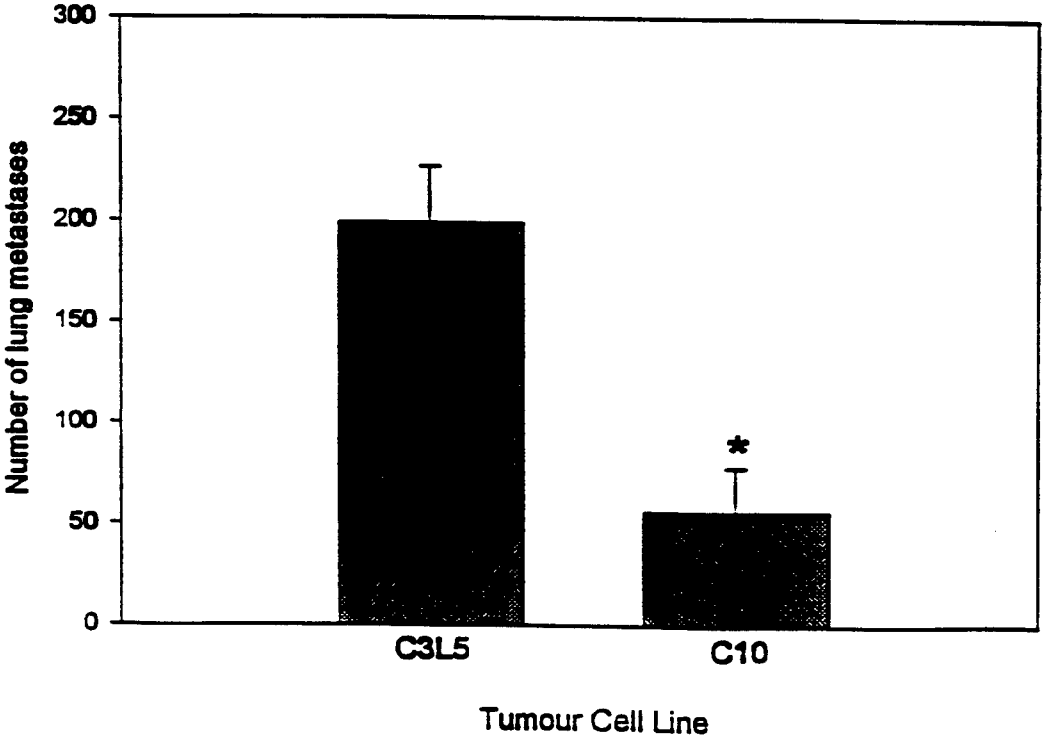

Number of lung metastases
300
250
200
150
100
50
0
C3L5
C10
Tumour Cell Line
*

Figure 1.

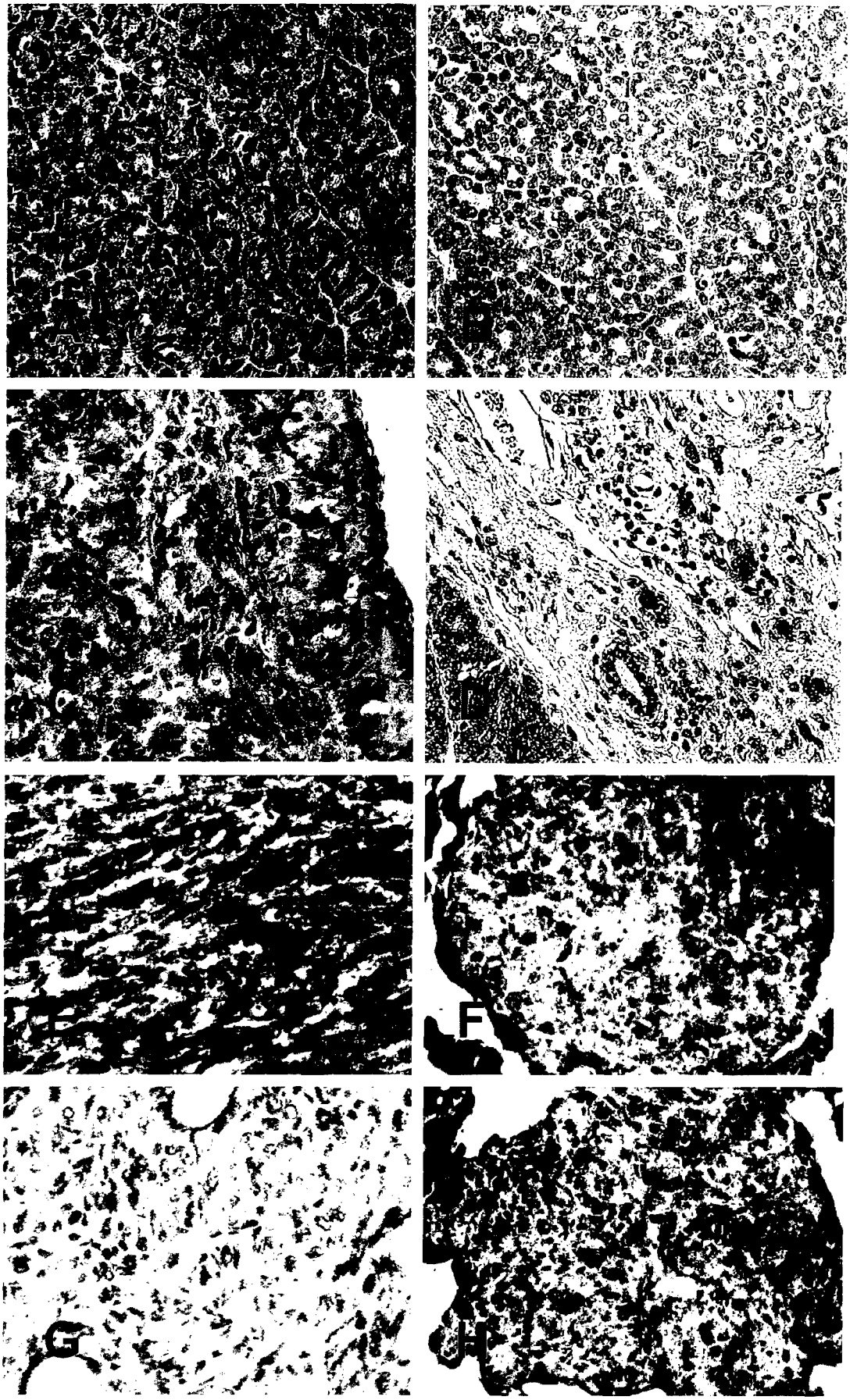


Figure 2

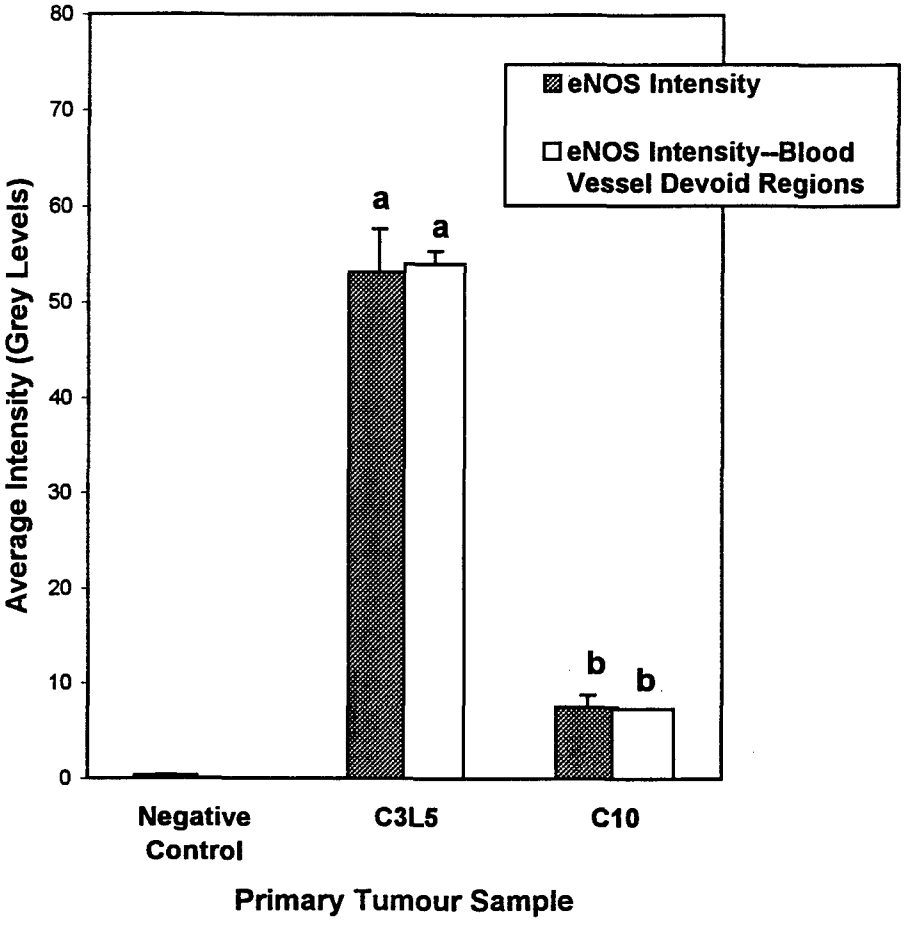

80
70
60
50
40
30
20
10
0
Average Intensity (Grey Levels)
eNOS Intensity
eNOS Intensity--Blood Vessel Devoid Regions
a
a
b
b
Negative Control
C3L5
C10
Primary Tumour Sample

Figure 3A

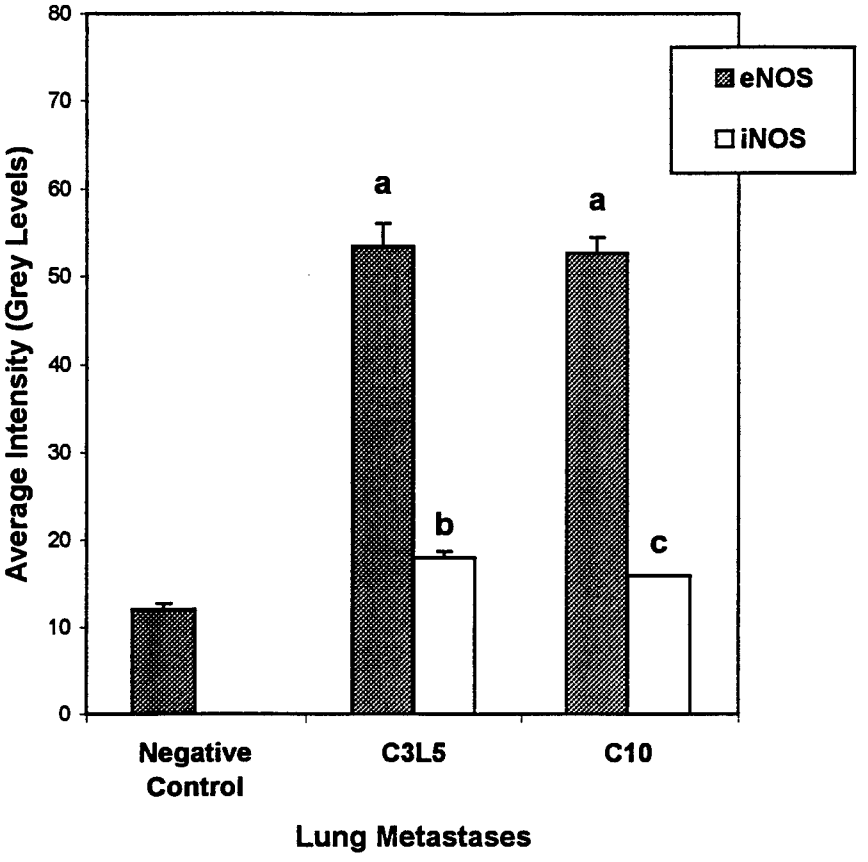
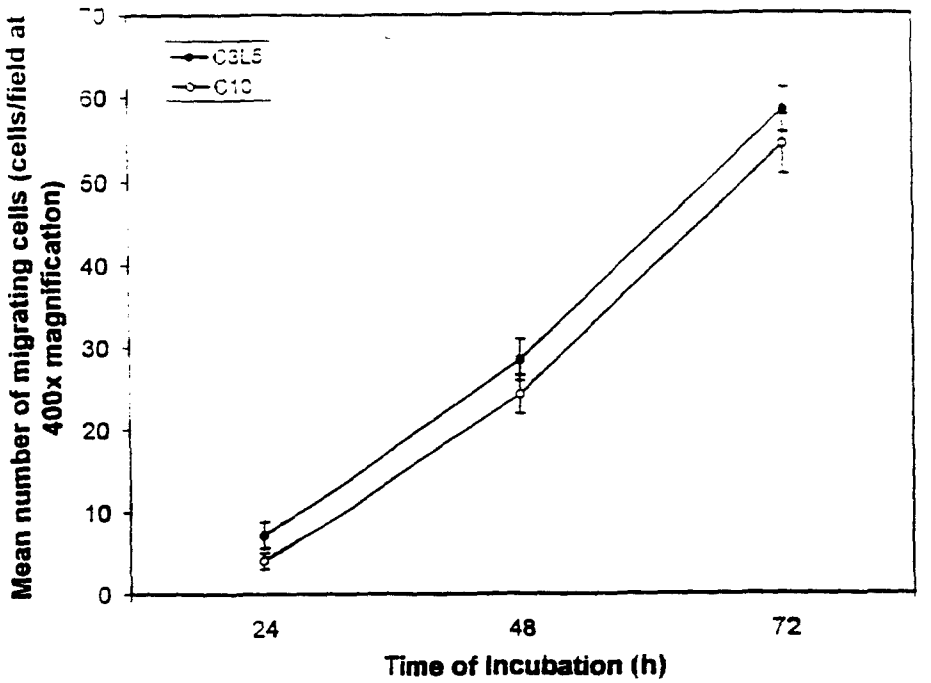

80
70
60
50
40
30
20
10
0
Average Intensity (Grey Levels)
eNOS
iNOS
a
a
b
c
Negative Control
C3L5
C10
Lung Metastases

Figure 3B


C3L5
C10
Mean number of migrating cells (cells/field at 400x magnification)
0
10
20
30
40
50
60
24
48
72
Time of Incubation (h)

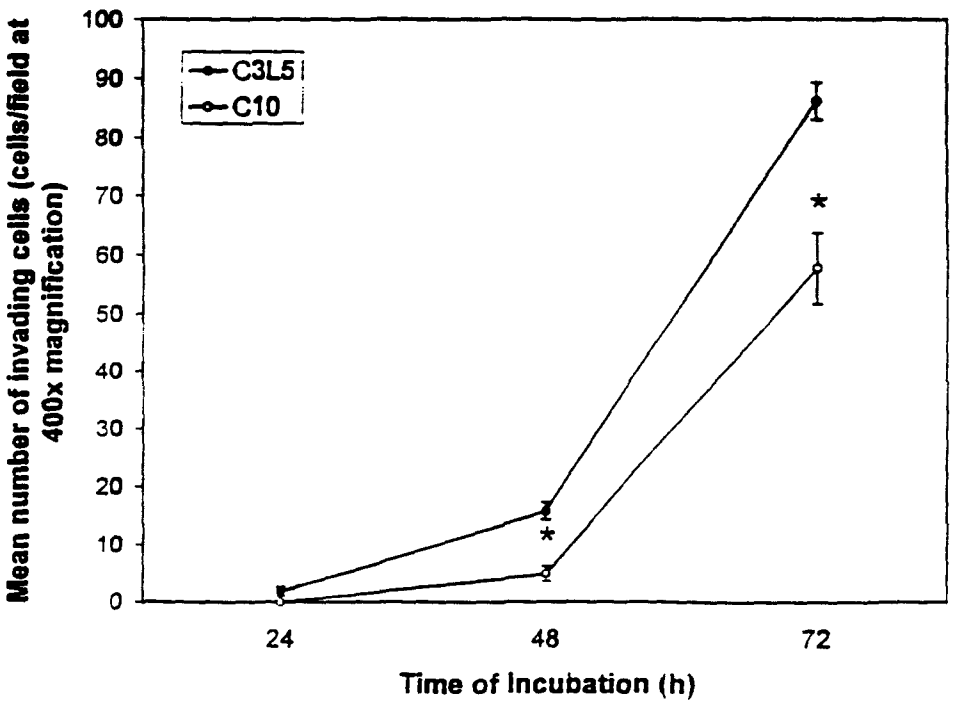

C3L5
C10
Mean number of invading cells (cells/field at 400x magnification)
0
10
20
30
40
50
60
70
80
90
100
*
*
24
48
72
Time of Incubation (h)

Figure 4

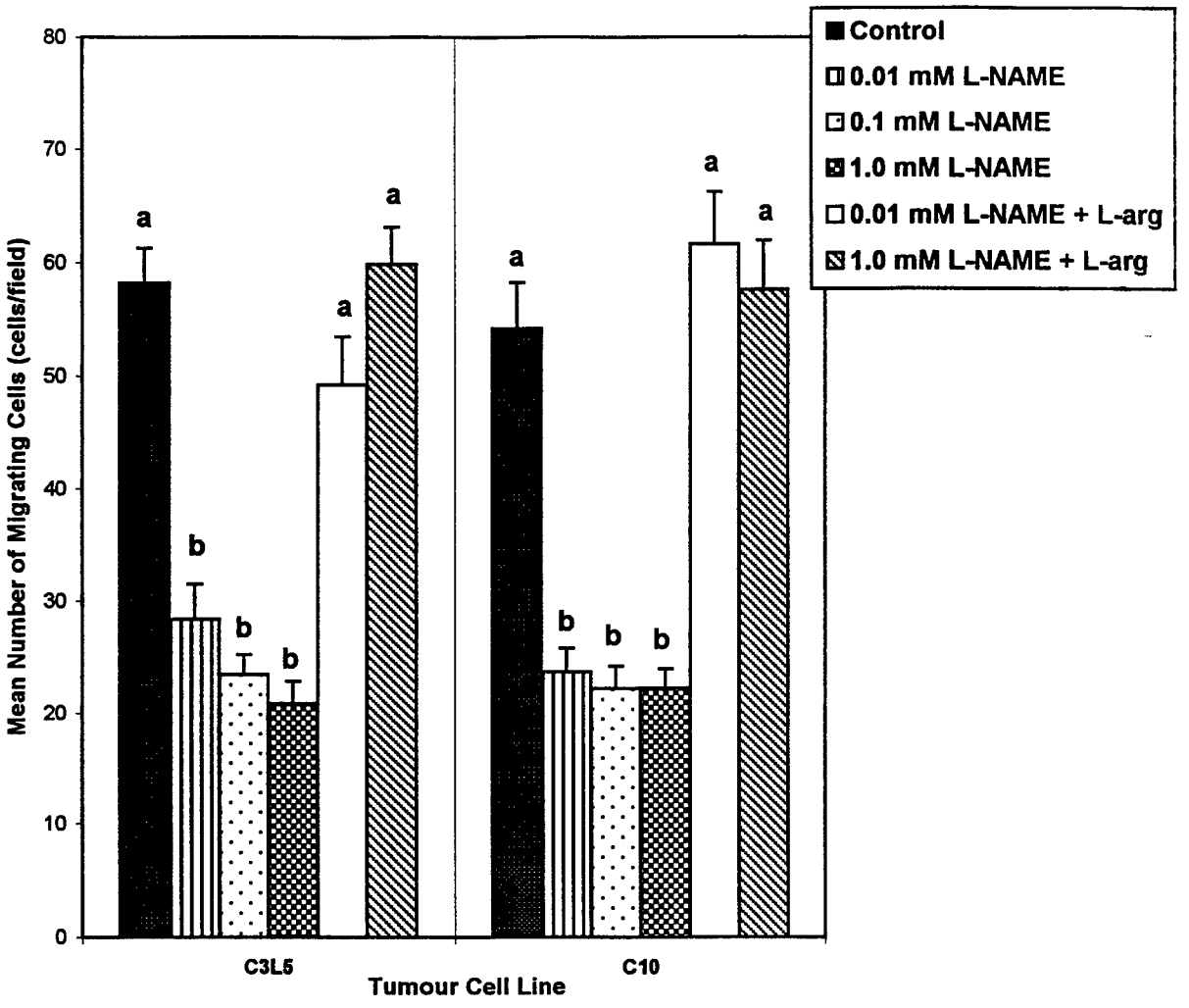

Control
0.01 mM L-NAME
0.1 mM L-NAME
1.0 mM L-NAME
0.01 mM L-NAME + L-arg
1.0 mM L-NAME + L-arg
Mean Number of Migrating Cells (cells/field)
0
10
20
30
40
50
60
70
80
a
b
b
b
a
a
a
b
b
b
a
a
C3L5
C10
Tumour Cell Line

Figure 5A

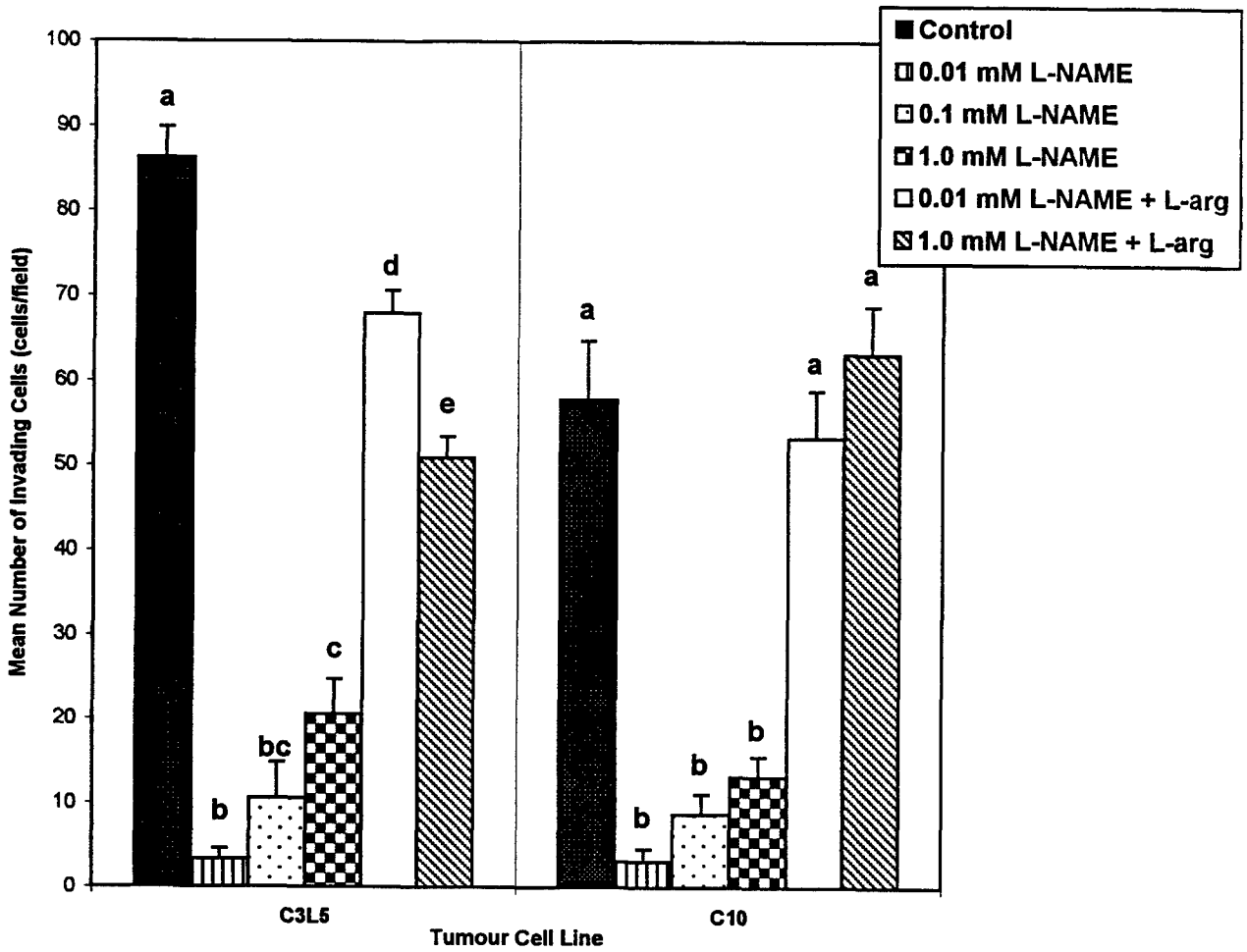

Control
0.01 mM L-NAME
0.1 mM L-NAME
1.0 mM L-NAME
0.01 mM L-NAME + L-arg
1.0 mM L-NAME + L-arg
Mean Number of Invading Cells (cells/field)
100
90
80
70
60
50
40
30
20
10
0
a
b
bc
c
d
e
a
b
b
a
a
C3L5
C10
Tumour Cell Line

Figure 5B

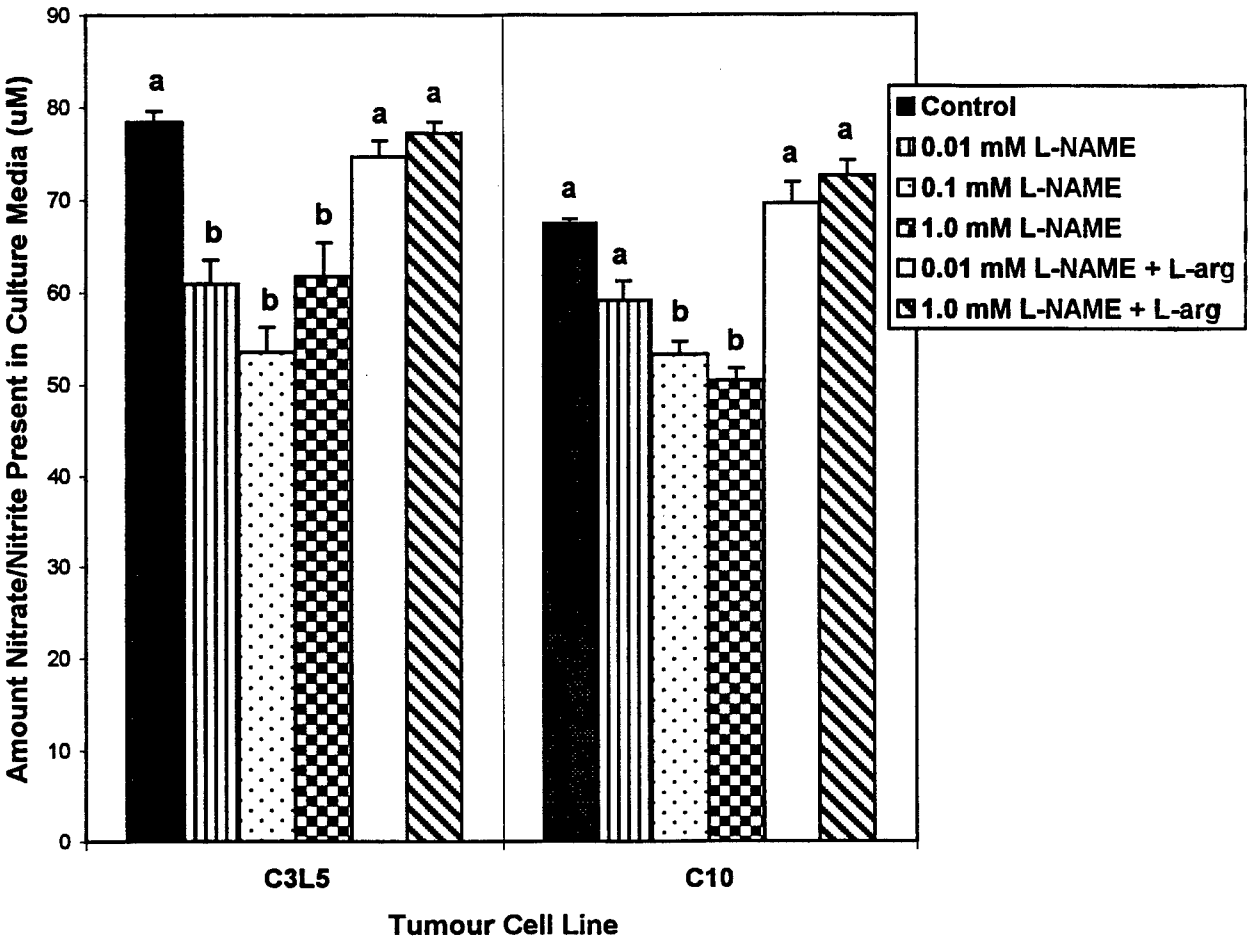

Amount Nitrate/Nitrite Present in Culture Media (uM)
90
80
70
60
50
40
30
20
10
0
a
b
b
b
a
a
a
a
b
b
a
a
C3L5
C10
Tumour Cell Line
Control
0.01 mM L-NAME
0.1 mM L-NAME
1.0 mM L-NAME
0.01 mM L-NAME + L-arg
1.0 mM L-NAME + L-arg

Figure 6

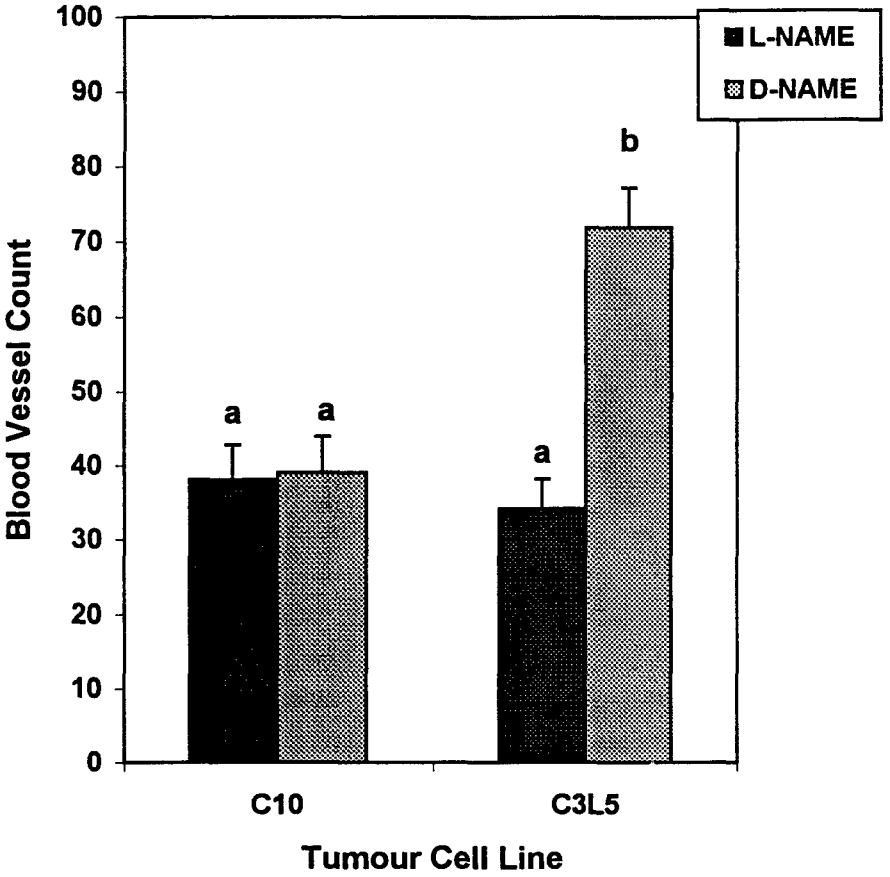

100
90
80
70
60
50
40
30
20
10
0
Blood Vessel Count
L-NAME
D-NAME
a
a
a
b
C10
C3L5
Tumour Cell Line

Figure 7

#3715 Nitric oxide synthases and murine mammary tumor progression.
Hum, K., Jadeski, L., and Lala, P.K. *University of Western Ontario, London, Ontario, Canada N6A 5C1.*

Our laboratory has previously found a positive correlation between the expression of nitric oxide synthase (NOS) and tumor progression; treatment with NOS inhibitors had antitumor and antimetastatic effects that were partly attributed to reduced tumor cell invasiveness. The present study examines the role of NO in tumor growth and metastasis in the C3H/HeJ murine mammary tumor model, utilizing spontaneously arising tumors, and clonal derivatives (C10 and C3L5) of a spontaneous C3H/HeJ tumor, which vary in their metastatic capacity; C10 is lowly metastatic, C3L5 is highly metastatic. Tumor cells within spontaneous mammary tumors heterogeneously expressed eNOS, lung metastases were homogenously eNOS positive; iNOS was expressed in tumor-associated macrophages at both sites, suggesting that eNOS promotes metastasis. Comparing C10 and C3L5 cells: 1) relative to C3L5 cells, primary site growth rate of C10 cells was slower, and number of pulmonary metastases was reduced. 2) Expression of eNOS *in vitro*, and at primary tumor sites, was higher for C3L5 relative to C10 cells. In both cases, tumor-associated macrophages, but not tumor cells, expressed iNOS. 3) A corresponding difference in *in vitro* invasiveness of these cells was observed; invasion of C10 and C3L5 cells was inhibited with L-NAME, and variably restored with excess L-arginine. These findings suggest that, 1) eNOS expression promotes primary tumor growth and metastasis, and 2) NO-mediated promotion of tumor growth and metastasis can be explained, in part, by the stimulation of tumor cell invasiveness. (Supported by US AMRAA, Grant # 1 96 6096)

NITRIC OXIDE (NO) SYNTHASE EXPRESSION PROMOTES GROWTH AND METASTASIS OF MURINE BREAST CANCER CELLS DUE TO INVASION AND MIGRATION-STIMULATION BY NO. Lala, P.K., Hum, K. and Jadeski, L. Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada N6A-5C1.
There is a positive correlation between nitric oxide synthase (NOS) expression/activity and tumor progression in many human and animal cancers including breast cancer. Primary spontaneous mammary tumors developing in C3H/HeJ female retired breeder mice immunostained positively for endothelial type (e) NOS, but significantly more tumor cells at metastatic lung sites expressed the enzyme, suggesting that eNOS expression was conducive to metastasis. This hypothesis was tested and verified in the transplanted tumor model using two tumor cell lines, C3L5 and C10, both clonally derived from a single spontaneous tumor. 1) C3L5 cells grew rapidly at primary subcutaneous sites, invaded the surrounding tissues and produced extensive spontaneous lung metastases, whereas C10 cells grew slowly at primary sites and metastasized to the lungs more slowly. 2) A corresponding difference in levels of eNOS protein expression (as revealed by immunostaining) in these cells was observed both *in vitro* and *in vivo* at primary tumor sites. However, their respective metastatic foci showed similar eNOS expression. iNOS was expressed in tumor-associated macrophages in both cases, but not in tumor cells. However, iNOS was equally inducible in both cell lines *in vitro* after LPS and IFN-γ treatment. 3) There was a corresponding difference in the invasive, but not migratory abilities, of these tumor cell lines *in vitro* when tested in transwells in the presence or absence of a Matrigel barrier in invasion and migration assays, respectively. However, the invasion and migration of both cell lines were inhibited in the presence of the NOS inhibitor L-NAME, and restored with the additional incorporation of excess L-arginine. Corresponding changes in NO production in the culture media as measured using the Greiss reaction indicated that tumor-derived NO had invasion and migration-promoting effects. These findings suggest that 1) eNOS expression is positively associated with tumor growth and metastasis in this tumor model, and 2) this association can be explained, at least in part, by NO-mediated stimulation of tumor cell invasiveness and migratory ability. (Supported by the US Army Grant # DMAD-17-96-6096).

Int. J. Cancer: **81,** 889–896 (1999)
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NITRIC-OXIDE PRODUCTION BY MURINE MAMMARY ADENOCARCINOMA CELLS PROMOTES TUMOR-CELL INVASIVENESS

Amila ORUCEVIC[1,2], John BECHBERGER[2], Angela M. GREEN[1], Richard A. SHAPIRO[1], Timothy R. BILLIAR[1] and Peeyush K. LALA[2]*

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The role of nitric oxide (NO) in tumor biology remains controversial and poorly understood. While a few reports indicate that the presence of NO in tumor cells or their micro-environment is detrimental for tumor-cell survival, and consequently their metastatic ability, a large body of data suggests that NO promotes tumor progression. The purpose of this study was to identify the source of NO in the spontaneously metastasizing C3-L5 murine mammary-adenocarcinoma model, the role of tumor-derived NO in tumor-cell invasiveness, and the mechanisms underlying the invasion-stimulating effects of tumor-derived NO. The source of NO was established by immunocytochemical localization of NO synthase (NOS) enzymes in C3-L5 cells *in vitro* and transplanted tumors *in vivo*. An *in vitro* transwell Matrigel invasion assay was used to test the invasiveness of C3-L5 cells in the presence or the absence of NO blocking agents or iNOS inducers (IFN-γ and LPS). The mechanisms underlying the invasion-stimulating effects of tumor-derived NO were examined by measuring mRNA expression of matrix metalloproteinases (MMP)-2 and -9, and tissue inhibitors of metalloproteinases (TIMP) 1, 2 and 3 in C3-L5 cells in various experimental conditions. Results showed that C3-L5 cells expressed high level of eNOS protein *in vitro*, and *in vivo*, both in primary and in metastatic tumors. C3-L5 cells also expressed iNOS mRNA and protein when cultured in the presence of IFN-γ and LPS. Constitutively produced NO promoted tumor-cell invasiveness *in vitro* by down-regulating TIMP 2 and TIMP 3. In addition, there was up-regulation of MMP-2, when extra NO was induced by IFN-γ and LPS. In conclusion, NO produced by C3-L5 cells promoted tumor-cell invasiveness by altering the balance between MMP-2 and its inhibitors TIMP-2 and 3. Thus, our earlier observations of anti-tumor and anti-metastatic effects of NO inhibitors *in vivo* in this tumor model can be explained, at least in part, by reduced tumor-cell invasiveness. *Int. J. Cancer* 81:889–896, 1999.
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Nitric oxide (NO) is synthesized in mammalian cells from the amino acid L-arginine by a family of enzymes, the nitric-oxide synthases (NOS) (Knowles and Moncada, 1994; Billiar, 1995). This molecule plays a key role in many physiological as well as pathological processes, including inflammation and neoplasia. Numerous clinical and experimental studies indicate a contributory role to NO in tumor progression. The level of NOS protein and/or NOS activity has been positively correlated with the degree of malignancy in a number of human cancers, including human gynecological cancers (ovarian, uterine) (Thomsen *et al.,* 1994), central nervous system tumors (Cobbs *et al.,* 1995), breast cancer (Thomsen *et al.,* 1995), gastric cancer (Thomsen and Miles, 1998), squamous-cell carcinomas of the head and neck (Gallo *et al.,* 1998), prostatic cancer (Klotz *et al.,* 1998) and lung cancer (Fujimoto *et al.,* 1997). Aberrant NOS expression in the above cases has been explained by the presence of constitutive form(s) (eNOS, nNOS) in tumor cells and/or tumor endothelial cells, or the expression of the inducible form (iNOS) in tumor endothelial cells and/or tumor associated macrophages. Expression of iNOS in the tumor neovasculature has also been reported in experimental tumors (Buttery *et al.,* 1993; Kennovin *et al.,* 1994). Moreover, several lines of direct evidence exist for a facilitatory role of NO in tumor progression: (a) in a rat colonic-adenocarcinoma model, treatment with N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, reduced NO production as well as tumor growth (Kennovin *et al.,* 1994); (b) similarly, anti-tumor and anti-metastatic effects of 2 NOS inhibitors, N^G-methyl-L-arginine (NMMA) and L-NAME, have been observed in our laboratory using a mouse mammary-adenocarcinoma model (Orucevic and Lala, 1996*a,b;* Lala and Orucevic, 1998); (c) Edwards *et al.* (1996) observed that NO induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in EMT-6 murine mammary-carcinoma cells stimulated tumor growth and metastasis *in vivo,* despite NO-induced inhibition of cell growth *in vitro;* (d) numerous human colon-cancer cell lines were found to express NOS activity (Jenkins *et al.,* 1994), and engineered over-expression of iNOS in a human colonic-adenocarcinoma line increased tumor growth and vascularity when transplanted into nude mice (Jenkins *et al.,* 1995).

In apparent contradiction to the above reports, the presence of NAD(P)H diaphorase, NOS enzymes and NOS activity in human colonic mucosa, polyps and carcinomas appeared to be inversely related to colonic-tumor progression (Chhatwal *et al.,* 1994; Moochhala *et al.,* 1996). However, a subsequent study revealed high iNOS expression in human colonic adenomas, consistent with the notion that this facilitated their progression into carcinomas by stimulating angiogenesis (Ambs *et al.,* 1998). High NOS activity was inversely correlated to tumor growth and metastasis in a murine melanoma model (Xie *et al.,* 1995). Engineered over-expression of iNOS in the above melanoma cells (Xie *et al.,* 1995) or human renal-carcinoma cells (Juang *et al.,* 1998) was shown to reduce tumorigenicity, because of NO-mediated tumor-cell apoptosis. These reported discrepancies may be explained by the dual role of NO on tumor growth. Whereas very high NO-producing tumor-cell clones may delete themselves by apoptosis, lower levels of NO may facilitate *in vivo* growth of surviving clones by numerous mechanisms, including promotion of neo-angiogenesis, increased tumor blood flow, increased invasiveness, or by inhibition of apoptosis. While some evidence exists for a contributory role of NO in promotion of neo-angiogenesis (Buttery *et al.,* 1993; Ziche *et al.,* 1994, 1997*a,b;* Lala and Orucevic, 1998; Gallo *et al.,* 1998) and tumor blood flow (Andrade *et al.,* 1992), the possible role of tumor-derived NO on invasiveness has not been explored.

The mechanisms by which NO promotes growth or metastasis in our murine mammary-adenocarcinoma model (Orucevic and Lala, 1996*a,b*) remained poorly defined. The objectives of the present study were to identify the source of NO in this spontaneously metastasizing C3-L5 mammary-adenocarcinoma model, its role on tumor-cell invasiveness and the mechanisms underlying the observed invasion-stimulating effects of NO.

Grant sponsor: Department of the United States Army; Grant number: DAMD 17-96-6069 (P.K.L.); Grant sponsor: NIHI; Grant number: GM 44100 (T.R.B.); Grant sponsor: Medical Research Council of Canada (A.O.).

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Received 30 July 1998; Revised 16 December 1998

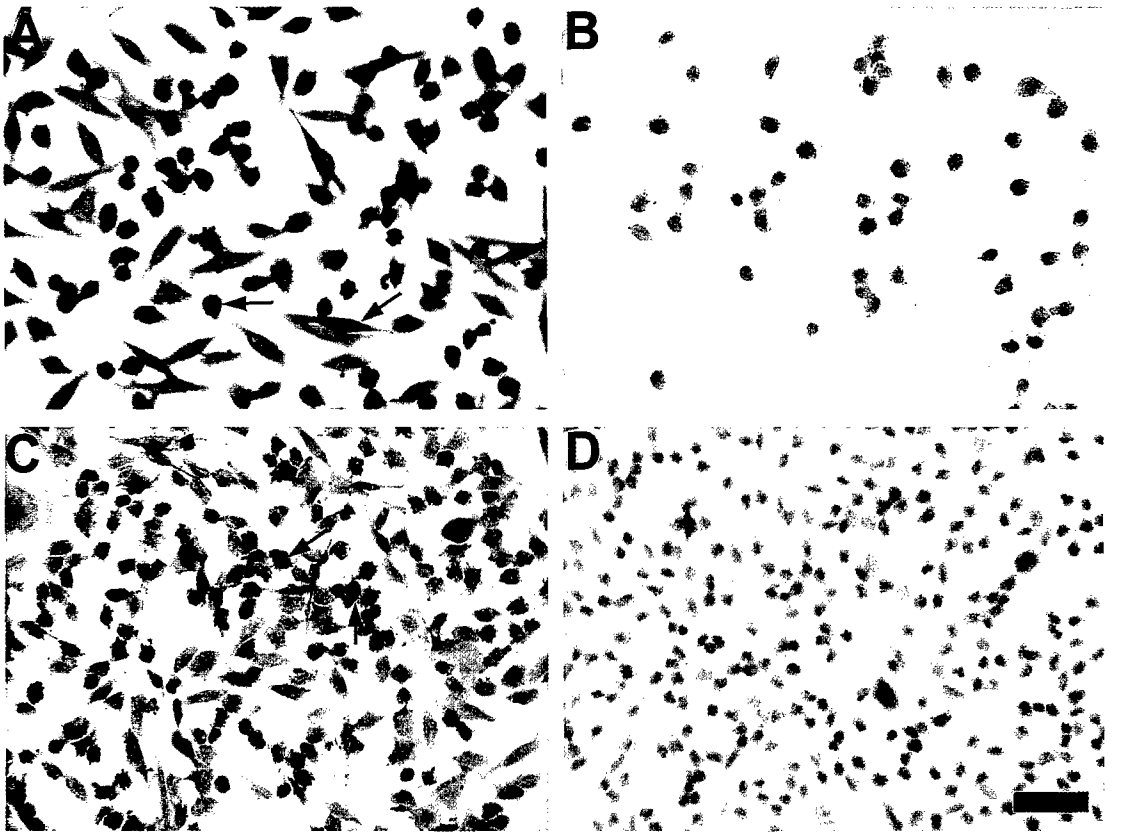

FIGURE 1 – Immunoperoxidase staining of C3-L5 cells in culture for NOS enzymes. *(a)* eNOS labelling is present in 90–95% of cells; *(b)* corresponding negative control (omission of primary antibody); *(c)* iNOS labelling is present in 20–25% of cells after induction with IFN-γ and LPS. Unstimulated cells showed no labelling for iNOS (data not shown); *(d)* corresponding negative control (omission of primary antibody). *(c)* and *(d)* were counterstained with hematoxylin. Scale bar: 50 μm.

MATERIAL AND METHODS

Tumor cell line and culture conditions

The C3-L5 mammary-adenocarcinoma cell line was selected and maintained in our laboratory from its parent C3 line, by 5 cycles of *in vivo* selection for spontaneous lung micrometastases following s.c. transplantation in C3H/HeJ mice (Lala and Parhar, 1993). C3 was a metastastic clone derived from a primary transplantable tumor T58, which had been isolated from a spontaneous mammary tumor in a C3H/HeJ retired female breeder mouse (Brodt *et al.*, 1985). Since the strong ability to spontaneously metastasize to the lungs, as originally exhibited by C3 cells, declined over the years of *in vitro* passages, re-selection was carried out *in vivo* to generate the C3-L5 line, which has since retained its ability to metastasize spontaneously to the lungs. C3-L5 cells were maintained in RPMI-1640 medium (GIBCO BRL, Burlington, Canada) with 1% penicillin/streptomycin (Mediatech, Washington, DC), supplemented with 10% FCS (GIBCO BRL). All experiments were done with cells passaged 3 to 5 times after thawing.

In experiments designed to block NO synthesis, L-NAME and NMMA (Sigma, St. Louis, MO) were added to the medium at various concentrations (0.01–1 mM); in some experiments, L-NAME was used at a concentration of 1 mg/ml of medium (equivalent to 3 mM). At all concentrations, there was no change in cell viability as measured by Trypan-blue exclusion.

In experiments designed to induce iNOS in tumor cells, a combination of recombinant murine IFN-γ (500–1000 U/ml) and LPS (10 μg/ml) was added to the culture medium. IFN-γ (lot FC2B11) was obtained from GIBCO, reconstituted with sterile water in aliquots of 10,000 U/100 μl and stored at −70°C until used for assays. LPS powder was obtained from Sigma, stored at 4°C, dissolved with complete medium on the day of the assay, and sterilized by filtration [0.2 μm filter pore size, Nalgene (Rochester, NY) syringe filters].

Immunocytochemical localization of NOS enzymes in C3-L5 cells in vitro

C3-L5 cells were grown for 24 hr on chamber slides, either in complete medium alone or in medium containing 500 U/ml of IFN-γ or 10 μg/ml of LPS or combination of IFN-γ and LPS in a humidified incubator (37°C, 5% CO_2 atmosphere). Slides were briefly washed with PBS, fixed in 10% buffered formalin and permeabilized with 0.25% Triton X-100 in PBS. After washing (3 × 5 min PBS), 10% normal goat serum was added to the slides as blocking serum for 1 hr. Slides were then subjected to the following treatments (30 min each) followed by washes: mouse monoclonal antibody against macrophage iNOS or endothelial NOS (eNOS) (Transduction Laboratories, Lexington, KY; 1:50 dilution), and secondary goat anti-mouse Ig biotinylated antibody (Dimension Laboratories, Mississauga, Canada; 1:200 dilution). Following treatment with ABC complex (1 hr) and DAB chromogen, slides were lightly counterstained with hematoxylin, and NOS expression was identified by positive brown staining. Negative controls were provided by omission of primary antibodies, or substitution of the primary antibodies by equivalent concentration of normal mouse Ig.

Immunohistochemical localization of NOS enzymes in C3-L5 cells in vivo

Samples of 5 primary tumors grown in C3H/HeJ mice for 24 days, following s.c. transplantation of 2.5×10^4 C3-L5 cells in the mammary line near the axilla, and of their spontaneous lung metastases were fixed in 10% buffered formalin, paraffin-embedded and cut into 5-μm-thick sections. After de-paraffiniza-

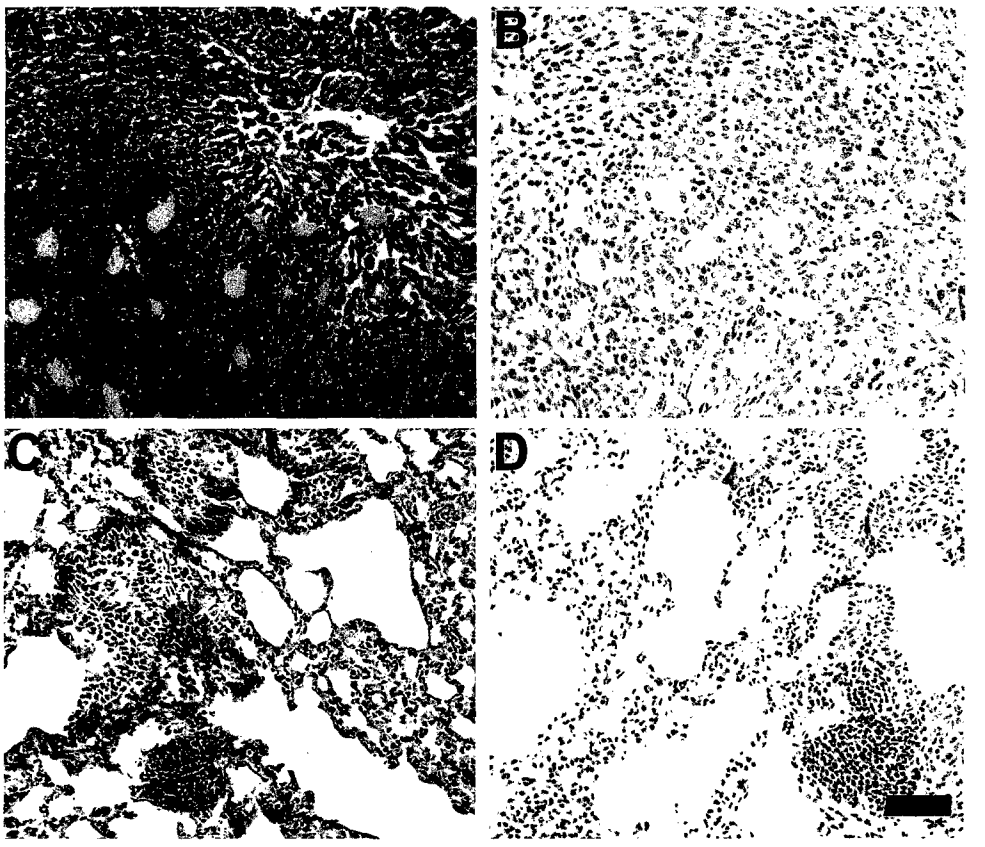

FIGURE 2 – Immunocytochemical localization of eNOS in primary tumors and their metastatic lung nodules, 24 days after s.c. transplantation of C3-L5 cells into C3H/HeJ mice. eNOS is present in approximately 80% of tumor cells at the primary site *(a)* and approximately 40–50% of tumor cells in a lung metastasis *(c)*. *(b)* and *(d)* are corresponding negative controls (omission of primary antibody). Sections were counterstained with hematoxylin. Scale bar: 50 μm.

tion and blocking of endogenous peroxidase activity (3% H_2O_2 in absolute methanol for 15 min), sections were permeabilized with 0.25% Triton X-100 in PBS. Normal horse serum (10%) or normal goat serum (10%) was added to the slides as blocking serum (1 hr), followed by mouse monoclonal primary antibody against iNOS or rabbit polyclonal antibody against eNOS (Affinity Bioreagents, Neshanic Station, NJ; 1:200 dilution) and incubated overnight at 4°C. Slides were then treated with secondary horse anti-mouse biotinylated antibody (rat absorbed IgG; Vector, Burlingame, CA; 1 in 200 dilution in 0.2% BSA in PBS) or goat anti-rabbit biotinylated antibody (as in earlier section) for 1 hr at room temperature, followed by ABC complex and DAB chromogen. Sections were then lightly counterstained with hematoxylin. Negative controls were provided by omission of primary antibodies, or substitution with normal mouse or rabbit Igs at equivalent concentrations.

Matrigel invasion by C3-L5 cells

An *in vitro* transwell Matrigel invasion assay devised in our laboratory (Graham *et al.*, 1993) was used to test the invasiveness of C3-L5 cells in the presence or absence of 0.01, 0.1, 1 and 3 mM NO blocking agent L-NAME or NMMA or iNOS inducers, IFN-γ (500–1000 U/ml) and LPS (10 μg/ml), in the presence or absence of L-NAME. Some wells contained excess L-arginine (5 times the concentration of NOS inhibitors, used as specificity control to abrogate the effects of NO inhibitors). In this assay, tumor cells were pre-labelled with ^{3}HTdR for 24 hr, then added to the invasion chamber of the transwell containing a millipore membrane coated with Matrigel (reconstituted basement membrane; Collaborative Research, Bedford, MA). Percentages of labelled cells penetrating the Matrigel-millipore membrane were scored as percent radioactivity appearing in the lower well and bottom of the millipore membrane, as a function of time (1–3 days). All assays were done in triplicate.

Measurement of NO production in the media from C3-L5 cells

C3-L5 cells were grown in 24-well plates (10^6 cells/800 μl media/well) in triplicate, in the medium alone, in L-NAME alone (1 mg/ml) or in the presence of IFN-γ (500–1000 U/ml) + LPS (10 μg/ml) ± L-NAME. Culture media from wells were collected after 24 hr of the incubation period, and kept frozen at −20°C, until assayed for NO_2^- levels. Griess reagent (Green *et al.*, 1982) was used for measurement of NO_2^-.

Analysis of mRNA expression for matrix metalloproteases 2 and 9 (MMP-2, MMP-9) and tissue inhibitors of metalloproteases (TIMP) 1, 2 and 3 in C3-L5 cells

C3-L5 cells were grown for 24 hr on 10-cm^2 tissue-culture dishes, either in RPMI medium with 1% BSA or in the medium with NMMA or IFN-γ + LPS ± NMMA. Total RNA was isolated by standard methods (Chomczynski and Sacchi, 1987) using RNAzol B (Biotecx, Houston, TX). Total RNA (20 μg) from each sample was electrophoresed on a 1% agarose gel containing 3% formaldehyde prior to transfer to Gene Screen membrane (DuPont-NEN, Boston, MA) and UV autocrosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA). cDNA probes for TIMP 1, 2 and 3 were obtained from Dr. R. Khokha (Ontario Cancer Institute, Toronto, Canada) and murine MMP-2 (72-kDa collagenase) and MMP-9 (92-kDa collagenase) probes were obtained from Dr. D. Edwards (University of Calgary, Canada). cDNA probe for murine

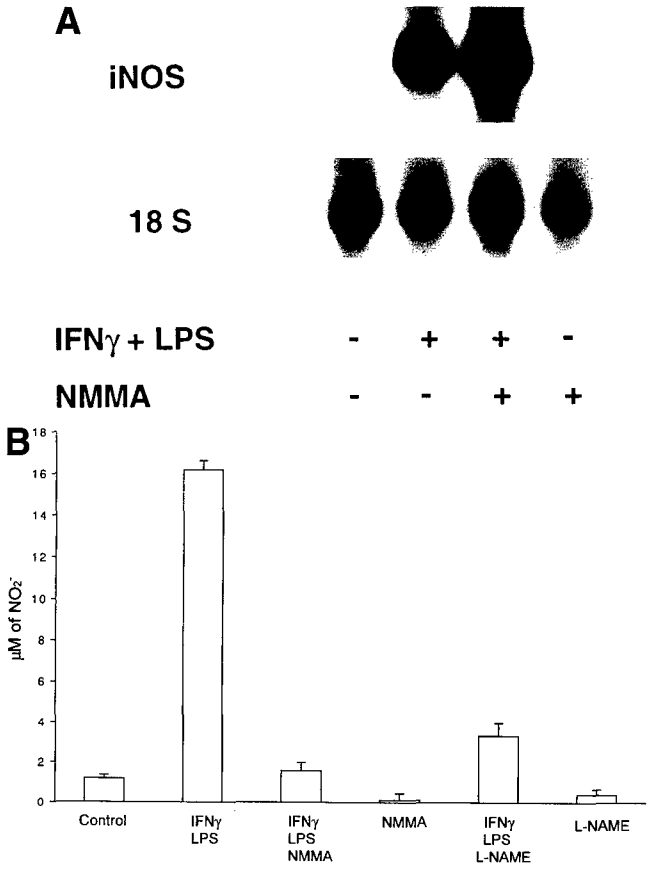

FIGURE 3 – iNOS induction and NO production following treatment of C3-L5 cells with IFN-γ and LPS. *(a)* mRNA expression of iNOS. iNOS was not expressed by C3-L5 cells in native condition, but was induced by IFN-γ and LPS. This induction was up-regulated by treatment of cells with NMMA. *(b)* NO_2^- levels in the medium of C3-L5 cells after 24-hr treatment with IFN-γ and LPS ± NMMA or L-NAME. Data represent mean (of triplicate determinations) ± S.E. IFN-γ and LPS treatment induced significant increase in NO production. Addition of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels.

iNOS was obtained from Dr. C. Lowenstain (Johns Hopkins University, Baltimore, MD). These cDNA probes were labeled with [^{32}P]dCTP by random priming. Hybridization was carried out overnight at 43°C and hybridized filters were washed at 53°C, following methods from published protocols (Geller *et al.,* 1995). Autoradiography was performed at −70°C in the presence of intensifying screen. Northern-blot membranes were stripped for re-hybridization with 18s rRNA utilized as loading controls. Relative mRNA levels were quantitated by PhosphorImager scanning using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

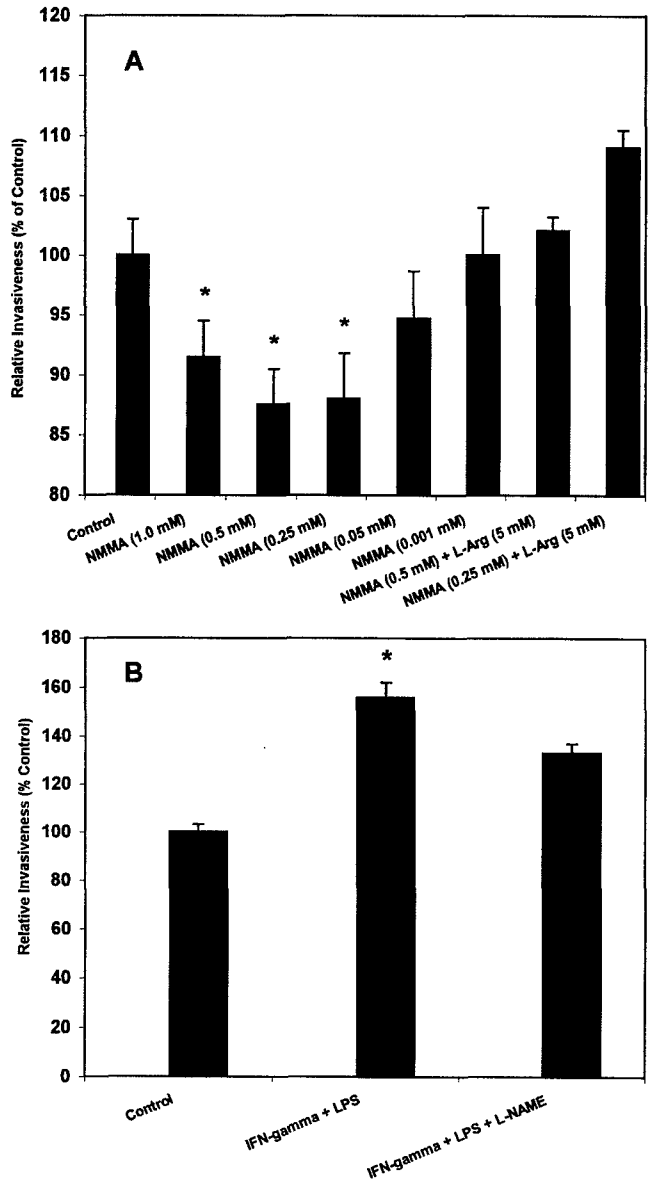

FIGURE 4 – Matrigel invasion by C3-L5 cells treated with different concentrations of NMMA (± L-arginine) *(a)* or IFN-γ and LPS (± L-NAME) *(b).* Data represent mean of triplicate determinations ± S.E. NMMA at concentrations of 0.25–1 mM reduced tumor-cell invasiveness ($p < 0.05$). L-arginine (5-fold excess) abrogated the NMMA effects, indicating the specificity of NMMA action. IFN-γ (500 U/ml) and LPS (1 mg/ml) significantly stimulated invasiveness of C3-L5 cells ($p < 0.05$). This stimulation was marginally abrogated ($p = 0.055$) by addition of L-NAME. *Significantly different ($p < 0.05$) from control.

FIGURE 5 – Northern blots of mRNA expression of MMP-2 and TIMP-1, 2, and 3 by C3-L5 cells in different conditions. Data indicate representative results from 1 of 2 separate experiments. MMP-9 (92-kDa collagenase) mRNA expression was absent in these cells (data not shown). *(a)* mRNA expression of MMP-2 (72-kDa collagenase). IFN-γ and LPS treatment up-regulated MMP-2 mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained collagenase-mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 expression. *(b)* mRNA expression of TIMP-1. IFN-γ and LPS treatment with or without NMMA, or NMMA treatment alone, did not significantly influence TIMP-1 mRNA expression. *(c)* mRNA expression of TIMP-2. IFN-γ and LPS treatment down-regulated TIMP-2 mRNA expression. Addition of NMMA to LPS and IFN-γ did not restore TIMP-2 expression. NMMA treatment alone, however, up-regulated expression of TIMP-2. *(d)* mRNA expression of TIMP-3. IFN-γ and LPS treatment down-regulated TIMP-3 mRNA expression. Addition of NMMA to LPS and IFN-γ partially restored TIMP-3 expression. NMMA treatment alone, however, up-regulated expression of TIMP-3.

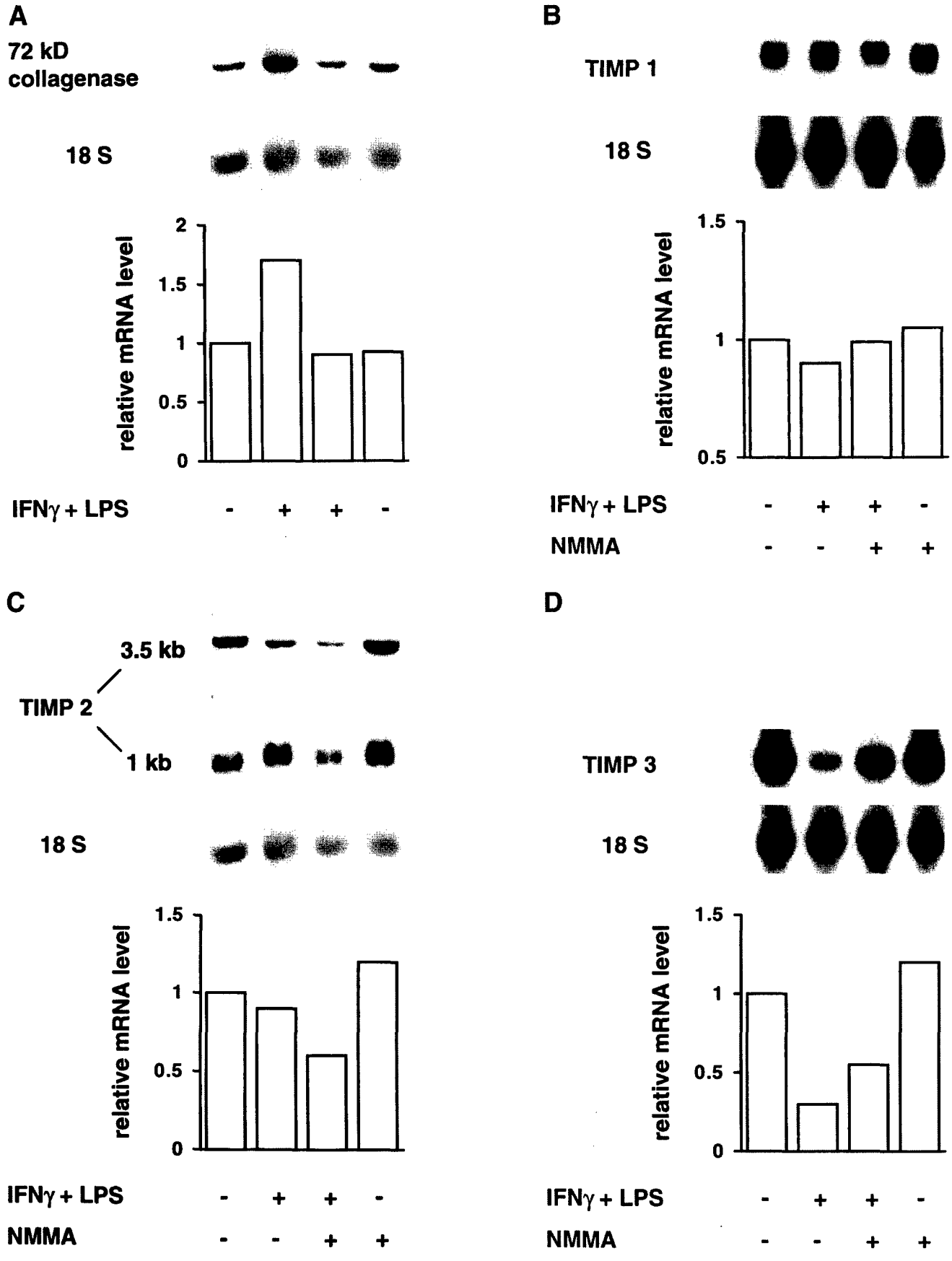
A
72 kD collagenase
18 S
relative mRNA level
2
1.5
1
0.5
0
IFNγ + LPS - + + -
B
TIMP 1
18 S
relative mRNA level
1.5
1
0.5
IFNγ + LPS - + + -
NMMA - - + +
C
3.5 kb
TIMP 2
1 kb
18 S
relative mRNA level
1.5
1
0.5
0
IFNγ + LPS - + + -
NMMA - - + +
D
TIMP 3
18 S
relative mRNA level
1.5
1
0.5
0
IFNγ + LPS - + + -
NMMA - - + +

FIGURE 5.

Statistics

Data were analyzed using one-way analysis of variance. Differences were considered significant at $p \leq 0.05$.

RESULTS

Expression of immunoreactive eNOS protein by C3-L5 cells in vitro *and* in vivo

Immunocytochemical staining for eNOS enzyme revealed that C3-L5 mammary-carcinoma cells constitutively expressed high levels of eNOS protein *in vitro* (Fig. 1*a*).

S.c. C3-L5 tumors grown in C3H/HeJ mice for 24 days, also their spontaneous metastatic counterparts (lungs) expressed eNOS protein (Fig. 2*a,c*). eNOS protein was present, on average, in approximately 80% of tumor cells at the primary tumor site and in about 40 to 50% of the tumor cells in the lung metastatic nodules harvested at 24 days, indicating that eNOS expression may be somewhat down-regulated during tumor growth at the metastatic site.

Expression of iNOS in C3-L5 cells in vitro

C3-L5 cells did not express iNOS mRNA under native conditions; however, iNOS mRNA was induced by 24-hr stimulation with IFN-γ and LPS (500 U/ml and 10 μg/ml, respectively). This induction was up-regulated by treatment of cells with NMMA (Fig. 3*a*). IFN-γ and LPS treatment induced significant increase in NO production, measured as NO_2^- levels in the medium. Additional presence of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels (Fig. 3*b*). Expression of immunoreactive iNOS protein after induction with the combination of IFN-γ and LPS (Fig. 1*c*) was correlated with mRNA expression. IFN-γ alone or LPS alone did not induce iNOS mRNA or protein in C3-L5 cells (data not presented).

Invasiveness of C3-L5 cells in vitro

C3-L5 cells, on their own, exhibited significant invasiveness in Matrigel invasion assay, as indicated by invasion indices ranging between 30 and 50% in different experiments. These values were normalized to 100% in Figure 4, to indicate the effects of NOS inhibitors (NMMA, L-NAME) or iNOS inducer (IFN-γ and LPS). NMMA at 0.25- to 1-mM concentrations reduced the invasiveness of C3-L5 cells. Addition of excess L-arginine abrogated the NMMA effects, indicating the specificity of NMMA action (Fig. 4*a*). Another NOS inhibitor, L-NAME, at concentrations of 0.01 to 1 mM also significantly reduced invasiveness of C3-L5 cells ($p < 0.05$) in a Matrigel invasion assay (data not shown).

Combination of IFN-γ and LPS (500 U/ml and 10 μg/ml respectively) significantly stimulated invasiveness of C3-L5 cells ($p < 0.05$) in a 3-day Matrigel invasion assay (Fig. 4*b*). Although the presence of L-NAME (1 mg/ml or 3 mM) did not abrogate this stimulation significantly, a trend was noted (Fig. 4*b*; $p = 0.055$). A combination of IFN-γ and LPS at higher concentrations (1000 U/ml and 10 μg/ml respectively), also significantly stimulated invasiveness of C3-L5 cells in 1-day and 3-day Matrigel invasion assays in separate experiments, in spite of the fact that at these concentrations there was a small drop in cell viability at day 3, most likely due to NO-mediated apoptosis (data not presented).

mRNA expression of MMPs and TIMPs in C3-L5 cells treated with IFN-γ and LPS, and with NMMA

IFN-γ and LPS treatment up-regulated MMP-2 (72-kDa collagenase) mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 mRNA expression (Fig. 5*a*). C3-L5 cells did not express MMP-9 (92-kDa collagenase) mRNA in any conditions (data not shown).

IFN-γ and LPS treatment with or without NMMA, or NMMA treatment alone, did not significantly influence TIMP 1 mRNA expression (Fig. 5*b*).

IFN-γ and LPS treatment caused minor down-regulation of TIMP-2 mRNA (Fig. 5*c*), and strong down-regulation of TIMP-3 mRNA (Fig. 5*d*) expression. Addition of NMMA to LPS and IFN-γ partially restored TIMP-3 expression (Fig. 5*d*), but TIMP-2 mRNA expression remained suppressed. NMMA treatment alone resulted in slight up-regulation of TIMP-2 and TIMP-3 mRNA expression (Fig. 5*c,d*).

DISCUSSION

Results from the present study revealed that *in vitro*-propagated C3-L5 mammary-adenocarcinoma cells expressed eNOS protein and, in addition, were stimulated to express iNOS protein, when grown in the presence of IFN-γ and LPS. Tumor cells grown *in vivo* expressed eNOS, but not iNOS protein, both at the primary site, as well as the sites of spontaneous lung metastasis. These cells exhibited a strong ability to invade Matrigel, and their invasiveness was reduced in the presence of NO-blocking agents (NMMA and L-NAME). This was paralleled by up-regulation of TIMP-2 and TIMP-3 mRNA expression. The anti-invasive effects of NOS inhibitors were noted in the absence of any change in cell viability (data not presented), and were abrogated in the presence of excess L-arginine, attesting to fact that the effects were due to inhibition of NO synthesis. Finally, invasiveness of C3-L5 cells was stimulated in the presence of iNOS-inducing agents IFN-γ and LPS, with concurrent expression of iNOS protein and increase in NO production *in vitro*. This was paralleled by up-regulation of MMP-2 mRNA and down-regulation of TIMP-2 and TIMP-3 mRNA. In this case, addition of NOS inhibitor failed to abrogate the invasiveness significantly but did, however, restore 72-kDa collagenase expression to the control level and only partially restored TIMP-3 but not TIMP-2 expression. Taken together, these results demonstrated that NO production by C3-L5 cells promoted tumor-cell invasiveness by altering the balance between expression of MMP-2 and its inhibitors TIMP 2 and TIMP 3. Further work is needed to demonstrate a corresponding shift in the activities of the enzyme and its inhibitors at the protein level.

We have reported (Orucevic and Lala, 1996,*a,b*) that treatment of C3H/HeJ mice bearing C3-L5 mammary-adenocarcinoma transplants with NOS inhibitors (NMMA and L-NAME) had significant anti-tumor and anti-metastatic effects. Growth-retarding effects of L-NAME were also observed in a rat colonic-adenocarcinoma model (Kennovin *et al.,* 1994), indicating that NO promoted tumor progression in these tumor models. Indeed, high NOS activity or NOS protein expression has been positively correlated to the progression of tumors of the human reproductive tract (Thomsen *et al.,* 1994), mammary gland (Thomsen *et al.,* 1995), stomach (Thomsen and Miles, 1998), central nervous system (Cobbs *et al.,* 1995), pharynx (Gallo *et al.,* 1998), prostate (Klotz *et al.,* 1998) and the lungs (Fujimoto *et al.,* 1997). In concurrence with these observations, the highly metastatic C3-L5 mammary-carcinoma cell line used in the present study was found to express high levels of eNOS protein *in vitro,* as well as *in vivo* in primary and metastatic tumors. C3-L5 cells also expressed iNOS protein following culture with IFN-γ and LPS. These findings attest to the NO-producing ability of these cells in constitutive conditions, which may be enhanced in inductive circumstances.

Multiple mechanisms may be postulated for the role of NO produced by tumor cells or host-derived cells in promoting tumor growth or metastases. Because of its vasodilatory function (Palmer *et al.,* 1987), NO may promote blood flow through the tumor vasculature and thus indirectly promote tumor-cell nourishment. This hypothesis has been validated in a rat adenocarcinoma model (Kennovin *et al.,* 1994), as well as in numerous other experimental tumors (Fukumura and Jain, 1998). NO has been shown to have a stimulatory effect on angiogenesis *in vitro* (Ziche *et al.,* 1994), as well as *in vivo,* when tested with a rabbit cornea model (Ziche *et al.,* 1994, 1997*a*), or a model of healing gastric ulcer (Konturek *et al.,*

1993). The angiogenesis-promoting role of NO has also been substantiated in a number of tumor models: (a) increased vascularity of transplants of human colonic-adenocarcinoma cells in nude mice, when these cells were engineered to over-express mouse iNOS gene (Jenkins *et al.,* 1995); (b) abrogation of C3-L5 murine mammary-tumor-induced angiogenesis in a Matrigel-implant assay, when the matrigel-implanted mice were subjected to L-NAME therapy (Lala and Orucevic, 1998; Jadeski and Lala, 1998); (c) reduction of angiogenesis in the rabbit cornea, induced by implantation of NO-producing human squamous-cell carcinomas when the rabbits were subjected to L-NAME therapy (Gallo *et al.,* 1998).

Another possible mechanism, direct NO-mediated stimulation of tumor-cell proliferation, has been excluded in our C3-L5 tumor model. Treatment of C3-L5 cells *in vitro* with NMMA had no effect on ^{3}HTdR uptake by these cells (data not shown). Finally, as demonstrated in this study, NO production by C3-L5 cells promoted tumor-cell invasiveness, and this mechanism may explain, at least in part, the observed reduction of primary tumor growth, as well as spontaneous lung metastasis following NMMA or L-NAME therapy (Orucevic and Lala, 1996*a,b*). Indeed, only modest anti-invasive effects of NOS inhibitors noted *in vitro* suggest that the *in vivo* anti-tumor action of the inhibitors in this tumor model must involve additional mechanisms such as reduced angiogenesis.

The mechanisms underlying the invasion-stimulating effects of NO in our tumor model appear to be, at least partly, due to an alteration in the balance between MMPs and their inhibitors. While endogenous, constitutive NO production by tumor cells caused down-regulation of TIMP-2 and TIMP-3, additional NO production under inductive conditions led to up-regulation of MMP-2. Such inductive conditions may occur *in vivo* during cytokine therapy of cancer and may counter some of the beneficial anti-tumor effects of cytokine therapy, such as tumor-cell death resulting from NO-mediated apoptosis or activation of anti-tumor effector cells.

Addition of NOS inhibitors to the NO inducer IFN-γ and LPS only partially abrogated C3-L5 tumor-cell invasiveness. There are several possible explanations: (a) incomplete inhibition of NO production, associated with sustained suppression of TIMP-2, in spite of restoration of MMP-2 expression to normal, as demonstrated in the results; (b) other NO-independent pathway(s) of invasion stimulation by IFN-γ and LPS.

Additional mechanisms underlying NO-mediated stimulation of cellular invasiveness may exist. For example, NO promotes degradation of articular cartilages by stimulating other MMPs (collagenases and stromelysin) in human, bovine or rabbit chondrocytes (Murrell *et al.,* 1995; Tamura *et al.,* 1996). In addition, it has been reported that TIMP-1 protein can be inactivated by peroxynitrite, which is formed rapidly from NO and O_2^- in conditions such as inflammation and ischemic re-perfusion (Frears *et al.,* 1996). Finally, NO has been shown to up-regulate urokinase-type-plasminogen activator (uPA) in endothelial cells of post-capillary venules during the process of NO-mediated stimulation of angiogenesis (Ziche *et al.,* 1997*b*). Since uPA converts plasminogen to plasmin, which can activate numerous MMPs, this may represent another pathway of NO-mediated stimulation of matrix degradation.

In apparent contrast to the above studies and the data presented here, engineered over-expression of iNOS in K1735 murine melanoma cells leading to decreased tumor-cell survival and tumorigenicity (Xie *et al.,* 1995) was reported to be associated with down-regulation of MMP-2 (Xie and Fidler, 1997) owing to down-regulation of its promoter activity. The reasons for this discrepancy remains unclear. Furthermore, additional biological roles of TIMP-1, TIMP-2 and TIMP-3 other than protease inhibition should not be disregarded. These molecules were reported to act as growth-promoting or -inhibiting proteins depending on the cell type (Bertaux *et al.,* 1991; Hayakawa *et al.,* 1992; Murphy *et al.,* 1993; Nemeth and Goolsby, 1993; Bian *et al.,* 1996; Sun *et al.,* 1996). Thus, further studies are needed to evaluate the biological consequences of NO-mediated regulation of MMPs and TIMPs in other tumor-cell systems. It is also possible that the genetic make-up of tumor cells may influence the biological role of NO in tumor progression (Ambs *et al.,* 1997; Lala and Orucevic, 1998). For example, expression of functional p53 in conjunction with iNOS was shown to promote NO-mediated apoptosis owing to *p53*-mediated accumulation of iNOS protein, whereas mutation or loss of *p53* provided a dual advantage to tumor cells: resistance to NO-mediated apoptosis, and increased tumorigenicity *in vivo* due to increased vascularity (Ambs *et al.,* 1997).

In conclusion, NO-mediated promotion of tumor-cell invasiveness resulting from an altered balance between MMP-2 and its inhibitors, in conjunction with the angiogenesis-stimulating role of NO, may account for the anti-tumor and anti-metastatic effects of therapy with NOS inhibitors in the C3-L5-mammary-adenocarcinoma model.

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THE ROLE OF NITRIC OXIDE IN MURINE MAMMARY TUMOR-INDUCED ANGIOGENESIS. Jadeski, L. and Lala, P.K. Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada N6A-5C1.

Using a murine breast cancer model, we earlier found a positive correlation between the expression of nitric oxide synthase (NOS) and tumor progression; treatment with inhibitors of NOS, N^G-Methyl-L-Arginine (NMMA) and N^G-Nitro-L-Arginine methyl ester (L-NAME), had antitumor and antimetastatic effects that were partly attributed to reduced tumor cell invasiveness. In the present study, we employed a novel *in vivo* model of tumor angiogenesis utilizing subcutaneous implants of tumor cells suspended in growth factor-reduced Matrigel to examine the angiogenic role of NO in a highly metastatic murine mammary adenocarcinoma cell line. This cell line, C3L5, expresses eNOS *in vitro* and *in vivo*, and iNOS *in vitro* upon stimulation with LPS and IFN-γ. Female C3H/HeJ mice received subcutaneous implants of growth factor-reduced Matrigel inclusive of C3L5 cells on one side and, on the contralateral side, Matrigel alone; L-NAME and D-NAME (inactive enantiomer) were subsequently administered for 14 days using osmotic minipumps. Immediately after sacrifice, implants were removed, and processed for immunolocalization of eNOS and iNOS proteins, and measurement of angiogenesis. Neovascularization was quantified in sections stained with Masson's trichrome or immunostained for the endothelial cell specific CD31 antigen. In addition, the mass of the implants and morphometric measurements of histologically distinct areas of peripheral tumor-free stroma, viable tumor tissue, and necrotic tissue were documented. Most tumor cells expressed eNOS, and positive immunoreactivity for iNOS protein was observed in endothelial cells and some macrophages within the tumor-inclusive implants. Measurable angiogenesis occurred only in implants containing tumor cells. Irrespective of the method of quantification employed, tumor-induced neovascularization was dramatically reduced in L-NAME-treated mice relative to those treated with D-NAME. The quantity of stromal tissue was lower, but the quantity of necrotic tissue higher in L-NAME relative to D-NAME-treated animals. The total mass of viable tissue (i.e., stroma and tumor cells) was lower in L-NAME relative to D-NAME-treated animals. These data suggest that NO is a key mediator of C3L5 tumor-induced angiogenesis and that the antitumor effects of L-NAME were partly mediated by reduced tumor angiogenesis. (Supported by the US Army Grant # DMAD 17-96-6096).

American Journal of Pathology. In press 1999

NOS INHIBITION BY N^G-NITRO-L-ARGININE METHYL ESTER (L-NAME) INHIBITS TUMOR-INDUCED ANGIOGENESIS IN MAMMARY TUMORS

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11 text pages, 3 figures
Running title: Nitric oxide Mediation of Tumor-induced Angiogenesis
key words: nitric oxide, angiogenesis, mammary tumor, eNOS, L-NAME
Supported by: US Army Grant DMAD-17-96-6096

Abstract

Using a murine breast cancer model, we earlier found a positive correlation between the expression of nitric oxide synthase (NOS) and tumor progression; treatment with inhibitors of NOS, N^G-Methyl-L-Arginine (NMMA) and N^G-Nitro-L-Arginine methyl ester (L-NAME), had antitumor and antimetastatic effects that were partly attributed to reduced tumor cell invasiveness. In the present study, we employed a novel *in vivo* model of tumor angiogenesis utilizing subcutaneous implants of tumor cells suspended in growth factor-reduced Matrigel to examine the angiogenic role of NO in a highly metastatic murine mammary adenocarcinoma cell line. This cell line, C3L5, expresses eNOS *in vitro* and *in vivo*, and iNOS *in vitro* upon stimulation with LPS and IFN-γ. Female C3H/HeJ mice received subcutaneous implants of growth factor-reduced Matrigel inclusive of C3L5 cells on one side, and on the contralateral side, Matrigel alone; L-NAME and D-NAME (inactive enantiomer) were subsequently administered for 14 days using osmotic minipumps. Immediately after sacrifice, implants were removed and processed for immunolocalization of eNOS and iNOS proteins, and measurement of angiogenesis. Neovascularization was quantified in sections stained with Masson's trichrome or immunostained for the endothelial cell specific CD31 antigen. While most tumor cells and endothelial cells expressed immunoreactive eNOS protein, iNOS was localized in endothelial cells and some macrophages within the tumor-inclusive implants. Measurable angiogenesis occurred only in implants containing tumor cells. Irrespective of the method of quantification employed, tumor-induced neovascularization was significantly reduced in L-NAME-treated mice relative to those treated with D-NAME. The quantity of stromal tissue was lower, but the quantity of necrotic tissue higher in L-NAME relative to D-NAME-treated animals. The total mass of viable tissue (i.e., stroma and tumor cells) was lower in L-NAME relative to D-NAME-treated animals. These data suggest that NO is a key mediator of C3L5 tumor-induced angiogenesis, and that the antitumor effects of L-NAME are partly mediated by reduced tumor angiogenesis.

Introduction

Nitric oxide (NO), an inorganic free radical gas, is synthesized from the amino acid L-arginine by a group of enzymes, the NO synthases (NOS). At least three isoforms of NOS have been cloned, characterized and localized: endothelial (e) and neuronal (n) NOS isoforms are Ca^{2+}/calmodulin-dependent, and are expressed constitutively in these and other cells. The inducible (i) isoform is Ca^{2+}/calmodulin-independent, and usually induced in the presence of inflammatory cytokines and bacterial products in macrophages, hepatocytes and other cells. Under certain conditions, iNOS can also be expressed constitutively in some cells. When constitutively expressed, NO produced at low levels is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation and neurotransmission. Under inductive conditions, high levels of NO produced by macrophages and other effector cells can mediate antibacterial and antitumor functions. However, chronic induction of NOS may contribute to many pathological processes including inflammation and cancer.[1,2]

Much scientific research has focused on the role of NO in tumor progression; although two apparently conflicting views exist, overall an overwhelming amount of clinical and experimental evidence supports a positive association between NO production and tumor progression. The level of NOS protein and/or activity in the tumor has been positively correlated with the degree of malignancy for tumors of the human reproductive tract,[3] breast,[4,5] and central nervous system[6]. In a majority of gastric carcinomas, iNOS was detected in stromal elements, and eNOS was detected in the tumor vasculature.[7] iNOS expression was higher in prostatic carcinomas relative to benign prostatic hyperplasia.[8] Similarly, relative to normal healthy control tissue, total NOS activity was higher in carcinomas of the larynx, oropharynx, oral cavity (9), and adenocarcinomas of the lung.[10]

Experimental tumor models have provided more direct evidence of a contributory role of NO in tumor progression. Using a rat adenocarcinoma model in which cells of the tumor vasculature expressed iNOS, treatment of the host with the NOS inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME) reduced NO production and tumor growth.[11] Furthermore, in spite of *in vitro* cytostatic effects of NO induction with lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine mammary cells, this induction stimulated tumor growth and metastasis *in vivo.*[12] Finally, in our own studies using a murine mammary adenocarcinoma model (C3H/HeJ spontaneous mammary tumors and their clonal derivatives), NO-

mediated stimulation of tumor progression was observed.[13] The spontaneously developing tumors showed heterogeneous expression of eNOS within primary tumors, whereas their metastatic counterparts were homogeneously eNOS positive, suggesting that eNOS expression promoted metastasis. A highly metastatic cell line, C3L5, clonally derived from a spontaneous mammary tumor showed strong eNOS expression *in vitro* and *in vivo*, and iNOS *in vitro* upon stimulation with LPS and IFN-γ.[13] Treatment of C3L5 mammary tumor-bearing mice with inhibitors of NOS, L-NAME and N^G-Methyl-L-Arginine (NMMA), had antitumor and antimetastatic effects.[14,15] Reduced tumor cell invasiveness was identified as one of the mechanisms mediating these effects.[13,16] We hypothesized that, in this tumor model, additional mechanisms likely played critical roles in mediating the therapeutic effects of NOS inhibition.

In contrast to the above, some studies reported an inverse association between NO and tumor progression. For example, the levels of NOS enzymes and NOS activity declined during the transition of human colonic mucosa to polyps, and then to carcinomas.[17] However, a later study revealed higher NOS activity in adenomatous polyps, which was believed to promote increased angiogenesis prior to the transition of adenomas into carcinomas.[18] In a murine melanoma cell line, NOS activity was inversely correlated with capacity for metastasis.[19] When genetically transduced to overexpress iNOS, the melanoma cells,[19] as well as renal carcinoma cells,[20] lost their tumorigenic and metastatic abilities, as a result of NO-mediated tumor cell apoptosis. These opposing findings suggest a dual role for NO in tumor growth and metastasis; the susceptibility of tumor cells to NO-mediated injury may depend on levels of NO produced, and the genetic makeup of the tumor cells. During clonal evolution of tumors, high NO-producing cells may self-delete by apoptosis, and those making lower levels of NO, or capable of resisting NO-mediated injury may have an *in vivo* advantage, resulting from NO-mediated stimulation of tumor cell invasiveness, tumor blood flow or tumor angiogenesis.[13]

A body of recent evidence suggests a stimulatory role of NO in angiogenesis. For example, NO donors were found to increase proliferation and migratory function of endothelial cells *in vitro.*[21,22] Using the *in vivo* rabbit cornea assay, angiogenesis induced by vasoactive molecules such as substance P and prostaglandin E $(PGE)_1$ was blocked with NOS inhibition.[22] Similarly, NOS inhibitors reduced neovascularization in acetic acid-induced gastric ulcers in rats,[23] and human squamous cell carcinoma xenografts in the rabbit cornea.[9] An angiogenesis-promoting role for tumor-derived NO was also suggested

by the *in vivo* behavior of a human colon adenocarcinoma cell line engineered to continuously express iNOS. When transplanted into nude mice, the iNOS-transduced cells resulted in tumors with enhanced growth rate and vascularity relative to those derived from wild type control cells.[24]

In the present study, we have evaluated the contributory role of NO in C3L5 mammary tumor-induced angiogenesis. To achieve this, we devised a novel *in vivo* Matrigel implant model of tumor-induced angiogenesis and subjected the host animals to chronic treatment with the NOS inhibitor L-NAME, or as controls, its inactive enantiomer, D-NAME.

Materials and Methods

Animals

Female C3H/HeJ mice (6-8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, Maine). Upon arrival at the vivarium, animals were immediately randomized to treatment groups (i.e., L-NAME and D-NAME); experimental procedures began after a one-week acclimatization period. Throughout the investigation, animals had free access to food (standard mouse chow) and water, and were maintained on a 12-hour light/dark cycle. Animals were treated in accordance with guidelines set out by the Canadian Council on Animal Care.

Tumor Cell Line

A spontaneously occurring mammary tumor in a female retired breeder C3H/HeJ mouse was the source of a primary transplantable tumor T58, from which a metastatic C3 cell line was derived. Since the metastatic potential of the C3 line declined over a number of years following repeated *in vitro* passages, a highly metastatic C3L5 line was derived by five cycles of repeated *in vivo* selections for spontaneous lung micrometastases following subcutaneous transplantation of C3 cells into C3H/HeJ mice.[25] The C3L5 cells used in the present study were grown from frozen stock and maintained in RPMI 1640 medium (GIBCO; Burlington, ON) supplemented with 5% fetal calf serum (GIBCO; Burlington, ON) and 1% penicillin-streptomycin (Mediatech; Washington, DC) in a humidified incubator, 5% CO_2.

In Vivo Assay for Tumor-induced Angiogenesis

We devised a novel *in vivo* model of tumor angiogenesis based on the protocol of Kibbey et al.[26] These authors utilized conventional Matrigel, a reconstituted basement membrane, which is liquid 4°C, and forms a solid gelatinous mass at body temperature. Measurable angiogenesis occurred within these implants, possibly due to angiogenic growth factors present in the conventional Matrigel. In our application of the assay we utilized growth factor-reduced Matrigel, which, unlike conventional Matrigel did not stimulate angiogenesis on its own. However, when tumor cells were suspended in growth factor-reduced Matrigel as a component of the subcutaneous implant, a strong angiogenic response was observed, which was easily, and objectively quantifiable. Based on several pilot experiments in which the implant volume, tumor cell number, and implant duration were varied, we standardized the assay (the detailed kinetics of tumor-induced angiogenesis in these implants are not presented here). This assay was used to examine the effects of NO on the angiogenic response by administering L-NAME or its inactive enantiomer, D-NAME, to mice using osmotic minipumps (pilot experiments established an equivalent angiogenic response in animals receiving D-NAME and those receiving no treatment). The angiogenic response was evaluated by a) examining the gross morphology of the Matrigel implants, and b) quantifying neovascularization in sections stained with Masson's Trichrome, or immunostained for the endothelial-specific CD31 antigen (PECAM). In addition, we documented the mass (weight in mg) of the implants upon retrieval, and systematically analyzed sections of tumor cell-inclusive implants for area quantification of three histologically distinct regions: a) peripheral tumor-free stromal tissue feeding blood vessels into the more deeply located tumor tissue, b) viable tumor tissue, and c) necrotic regions, to determine the effects of therapy on the various components of the implants.

In the inguinal region, mice received subcutaneous implants of 5×10^4 C3L5 cells suspended in Growth Factor Reduced Matrigel® (Collaborative Research, Bedford, MA) (3.5 mg Matrigel in 0.5 ml RPMI), and on the contralateral side as controls, the equivalent amount of Matrigel alone. Immediately thereafter, osmotic minipumps (©ALZA Corporation, Palo Alto, CA) were implanted subcutaneously, providing a constant systemic supply (0.5 ml per hour; 25 mg per 200 μl 0.9% NaCl) of L-NAME to one group (n = 15), or D-NAME to the other group (n = 15) (both drugs purchased from Sigma Chemical Co., St. Louis, MO) for

the experiment duration (14 days). This experiment was performed on two separate occasions; both experiments were conducted using the same protocol and sample size (i.e., n = 15 animals per group).

Mice were sacrificed using an overdose of pentobarbital, and Matrigel implants removed, divided in half, therefore paraffin and frozen sections were obtained from the same sample. Samples fixed in 4% paraformaldehyde, processed for paraffin embedding and sectioned were stained with Masson's Trichrome, or immunostained for eNOS and iNOS proteins. Alternatively, samples frozen in O.C.T. were sectioned and analyzed immunohistochemically for CD31. Both types of sections (i.e., Masson's Trichrome stained or CD31 immunostained) were scanned at low power for areas containing new blood vessels (researcher blind to experimental condition); these areas were systematically imaged at 160X magnification using Northern Exposure (Empix Imaging Inc.), and individual vessel counts for each field were documented using Mocha™ Image Analysis Software (Jandel Scientific) to identify fields of maximum blood vessel density (i.e., 'hot spots'). Subsequently, 'hot spots' were statistically analyzed for between-group differences using two different approaches: a) the average (n = 15 animals per group) of the maximal number of blood in one field per animal, and b) the average (n = 15 animals per group) of the average of three fields of maximal blood vessel density (taken in descending order) per animal. Masson's Trichrome-stained sections were also used to quantify histologically distinct regions within implants (i.e., peripheral stromal, healthy tumor, and necrotic regions); entire cross sections of the implants were digitally imaged; areas were then quantified, and data expressed as the number of pixels, using Mocha™ Image Analysis Software (researcher blind to experimental condition).

Immunohistochemical Localization of CD31 Antigen (PECAM-1)

O.C.T.-fixed samples were stored at -80°C until sectioned; samples were sectioned at 5μm thickness and stored at -20°C before immunostaining (sections stored for maximum of 2 days at -20° C). Frozen sections were fixed in ice-cold methanol (5 minutes, -20°C). Endogenous peroxidase activity was blocked with methanol containing 3% H_2O_2 (30 minutes, room temperature) prior to application of blocking serum: normal mouse serum (Cedarlane Laboratories Limited, Hornby, Ontario) diluted in 1% Bovine Serum Albumin (1:10; 1 hour at room temperature in humidified chamber). Sections were then incubated with primary antibody: purified rat anti-mouse CD31 monoclonal antibody (1:50; overnight at 4°C in humidified

chamber; Cedarlane Laboratories Limited, Hornby, Ontario) followed by secondary antibody: biotinylated mouse anti-rat IgG-2a monoclonal antibody (1:100; 1 hour at room temperature in humidified chamber; Caltag Laboratories, San Francisco, CA). Avidin-biotin complex (ABC) (Vector Laboratories, Inc., Burlingame, CA) was then applied (1 hour at room temperature), followed by diaminobenzidine (DAB) chromogen (Sigma Chemical Company, St. Louis, MO); sections were then lightly counterstained with Mayer's Haemalum. Negative controls were incubated with the equivalent concentration of rat IgG-2a (Caltag Laboratories, San Francisco, CA) in place of primary antibody.

Immunohistochemical Localization of eNOS and iNOS Antigens

Paraffin embedded implants were sectioned at 7 μm thickness. Following deparaffinization and rehydration of sections, endogenous peroxidase activity was blocked using methanol containing 3% H_2O_2 prior to application of blocking serum: normal horse serum (1:10; 1 hour at room temperature). Sections were then incubated with primary antibody: mouse monoclonal anti-eNOS or mouse monoclonal anti-macrophage iNOS (1:80; overnight at 4°C, or 1:50; overnight at 4°C; Transduction Laboratories, Lexington, KY) for eNOS and iNOS localization, respectively. Secondary antibody: biotinylated horse anti-mouse (1:200; 1 hour at room temperature) was then applied, followed by ABC (1 hour at room temperature) and DAB chromogen. Sections were lightly counterstained with Mayer's Haemalum.

Data Analysis

Data were analyzed using SAS v6.12 on a Unix mainframe, and treatment groups (i.e., L-NAME and D-NAME) compared using one way analysis of variance (ANOVA). In quantifying neovascular response for each treatment group (n = 15 mice per group), results were expressed as a) the mean of the maximum number of microvessels in a single field (160X magnification), and b) the mean number of microvessels in three fields of maximum blood vessel density (160X magnification). Data from the duplicate experiment (n = 15 mice per group) were analyzed in the same manner. A probability of 0.05 was used in determining statistical significance.

Results

Gross Morphology of Implants

Figure 1 shows the gross morphology of tumor-exclusive implants (Figure 1A), and tumor-inclusive implants obtained from L-NAME and D-NAME-treated (Figures 1B and 1C, respectively) animals. Tumor-exclusive implants from L-NAME and D-NAME-treated animals were small, translucent and avascular. Tumor-inclusive implants were larger, and implants obtained from L-NAME-treated animals were less vascular than those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—Masson's Trichrome Staining

Figure 1 shows Masson's Trichrome staining of tumor-exclusive Matrigel implant (Figure 1D) (sections of tumor cell-exclusive implants were identical for L-NAME and D-NAME-treated animals), and tumor-inclusive implants obtained from L-NAME (Figure 1E) and D-NAME-treated animals (Figures 1F). This method stains fibrous tissue and stroma bluish-green. Blood vessels containing red blood cells stand out because of bright red staining of red blood cells. Other cells (including tumor cells) show pink staining of cytoplasm and dark magenta colored nuclei. Tumor-exclusive Matrigel implants obtained from L-NAME and D-NAME-treated animals were avascular and contained a few fibroblasts. Tumor-inclusive implants obtained from both treatment groups consisted of three histologically distinct regions, shown in Figure 1G (implant obtained from L-NAME-treated animal): a) a peripheral zone of stroma (*S*) containing feeder blood vessels, b) healthy tumor areas (*T*), and c) more centrally located, necrotic areas (*N*) infiltrated with leukocytes. Areas of highest microvascular count were best identified in the stroma of tumor-inclusive implants in Masson's Trichrome-stained sections. The stroma of tumor-inclusive implants obtained from L-NAME-treated animals appeared thinner and less vascular relative to those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—CD31 Immunostaining

Figure 2 shows immunohistochemical localization of CD31 antigen in implants obtained from D-NAME and L-NAME-treated animals (Figures 2A and 2B, respectively). This method stains endothelial cells brown, and correctly identifies cells lining the microvasculature within the tumor component of the implants (in contrast to Masson's Trichrome-stained sections, which predominantly identifies blood vessels within stromal areas).

Nuclei are lightly counterstained with Mayer's Haemalum. Neovascularization was reduced in tumor-inclusive implants obtained from L-NAME-treated animals relative to those obtained from D-NAME-treated animals.

Histological Evaluation of Implants—eNOS and iNOS Immunostaining

Immunohistochemical localization of eNOS antigen in tumor-inclusive implant, and a negative control are shown in Figures 2C and 2D, respectively. eNOS expression was observed in endothelial cells of the tumor vasculature (Figure 2C, inset), and a high proportion of tumor cells within implants obtained from animals treated with either L-NAME or D-NAME. Immunohistochemical localization of iNOS antigen is shown in Figures 2E and 2F. Regardless of treatment group, tumor cells within the implants did not stain positively for iNOS. However, positive immunoreactivity for iNOS protein was observed in a significant proportion of macrophages located in peripheral stroma (Figure 2E), in the healthy tumor bordering the necrotic area (Figure 2F), and within the central necrotic area (not shown in Figure 2). Endothelial cells also stained positively for iNOS (Figure 2E, small arrow).

Quantification of Tumor-induced Neovascularization—Masson's Trichrome Staining and CD31 Immunostaining

Figure 3 shows the number of blood vessels per unit area for Masson's Trichrome-stained and CD31 immunostained sections; data are expressed as the maximum number of blood vessels per field and as the average number of blood vessels in three fields of maximal density. Irrespective of staining protocol or method of quantification utilized, the neovascular response was reduced in L-NAME-treated mice relative to those treated with D-NAME. Tumor-induced neovascularization, measured by Masson's Trichrome, was reduced in implants obtained from L-NAME-treated animals when data were expressed as 1) the maximum number of blood vessels per field (L-NAME, 52.3 $\pm$ 5.9; D-NAME, 92.3 $\pm$ 11.3, $P < 0.003$), and 2) as the average number of blood vessels in three fields (L-NAME, 42.1 $\pm$ 5.4; D-NAME, 83.2 $\pm$ 10.2, $P < 0.001$) (Figures 3A and 3B, respectively). L-NAME treatment reduced tumor-induced neovascularization, as measured by CD31 immunostaining, when data were expressed 1) as the maximum number of blood vessels per field (L-NAME, 50.8 $\pm$ 7.6; D-NAME, 79.9 $\pm$ 6.5, $P < 0.0099$) and 2) as the average number of

blood vessels in three fields (L-NAME, 69.6 ± 4.8; D-NAME, 40.1 ± 5.6, $P < 0.0009$) (Figures 3C and 3D, respectively). Results obtained from the duplicate experiment were similar, thus are not presented.

Quantification of Histologically Distinct Areas within Implants

Quantification of the various tissue compartments contained within tumor cell-inclusive implants indicated that the quantity of peripherally located stromal tissue was reduced in L-NAME relative to D-NAME-treated animals (L-NAME, 4998.5 ± 1055.2 pixels; D-NAME, 9758.3 ± 1515.4 pixels, $P < 0.02$), and the quantity of necrotic tissue was higher in L-NAME relative to D-NAME-treated animals (L-NAME, 73709.5 ± 8638.0 pixels; D-NAME, 38434.5 ± 7918.3 pixels, $P < 0.007$). In addition, the mass of viable tissue (i.e., stroma and tumor cells), was lower in L-NAME relative to D-NAME-treated animals (L-NAME, 17.1± 3.5%; D-NAME, 28.8 ± 4.0%, $P < 0.04$). Values for the mass of viable tissue were calculated using the following formula: weight of implant (mg) X (1 - necrotic fraction of the implant).

Discussion

Angiogenesis, the development of new blood vessels from the preexisting vascular bed is an essential feature of many physiological conditions including wound healing, embryonic development and endometrial proliferation. Numerous pathological conditions, such as diabetic retinopathy, rheumatoid arthritis and tumor growth are also characterized by abnormal neovascularization. Growth of solid tumors cannot proceed beyond a microscopic size without the development of an extensive vascular system.[27] Furthermore, because the degree of vascularization often correlates with poor clinical prognosis and increased likelihood of metastasis of a number of human tumors,[28,29] targeting angiogenesis in the therapeutic intervention of cancer has received substantial recent attention. Although a number of compounds characterized as inhibitors of angiogenesis have entered clinical trials, intense efforts to identify potent angiogenesis inhibitors with improved selectivity continue. However, a consistent limitation of these investigations has been the availability of simple, reproducible, reliable and easily quantifiable assays that reflect *in vivo* systems for tumor-induced angiogenesis. Overly simplistic cellular *in vitro* systems, and technical difficulties of currently used *in vivo* angiogenesis assays are important limiting factors.

In vitro models of angiogenesis[22,30] may not be ideal for measuring tumor-induced angiogenesis because of the inability in providing all the necessary cells and/or factors that may interact in the *in vivo* tumor environment. The most widely used *in vivo* systems are the rabbit cornea and the chick chorioallantoic membrane (CAM) assays, in which variability of results and subjectivity in quantification remain important limitations.[31] A major concern of the rabbit cornea assay is the potential development of xenograft reactions after tumor implantation. Although the cornea is an immunoprivileged site, as it gradually becomes vascularized, the possible contribution of xenograft reactions to the angiogenic response cannot be disregarded. The CAM assay does not present this problem, because the host is naturally immunodeficient. However, major disadvantages of this assay are the time limit of 7-10 days imposed by embryo growth and acquisition of immunocompetence,[32] and difficulties in objectively quantifying the neovascular response.[31,33] Another potential concern is inherent with properties of the chick chorioallantoic membrane, which is a growing and developing embryonic structure; the relative contribution of vasculogenesis and/or angiogenesis, two discrete processes which are differentially regulated, to the development of new blood vessels may not be clear.

The present *in vivo* model of tumor-induced angiogenesis is devoid of these limitations. The assay is simple, reproducible, and objectively quantifiable. Suspension of tumor cells within the Matrigel matrix serves to effectively immobilize tumor cells; the ensuing angiogenic response is organized, and, all stages of the neovascular response can be quantified. Therefore, the kinetics of tumor development and neovascularization may be followed for a considerable length of time (e.g., 2 weeks in the present experiment); in separate experiments, we have established that the experimental procedure can be extended (e.g., up to 6 weeks). Furthermore, because the developing blood vessels converge within a discrete area, retrieval and quantification of developing blood vessels are simple and complete. Use of a specific inoculation site in the murine model minimizes variation of the angiogenic response. Although we utilized an inbred mouse strain and its syngeneic tumor, nude mice could appropriately serve as the host for xenografts in other applications of this assay, in particular, for human tumors.

We utilized the present assay to evaluate the effects of NO on the angiogenic response by administering L-NAME or D-NAME to mice using osmotic minipumps in two separate experiments. Results from both clearly showed that, irrespective of method of quantification, NOS inhibition dramatically reduced

the neovascular response. The growth patterns of histologically distinct areas present within the implants were differentially affected by NOS inhibition. The stromal component of the implants, which supports the vascular supply, was reduced in L-NAME relative to D-NAME-treated animals. In addition, there was increased necrosis and reduced viable tissue mass within implants obtained from L-NAME relative to D-NAME-treated animals, supporting prior observations of antitumor effects of NOS inhibition in mice transplanted with C3L5 mammary adenocarcinomas.[14,15] Therefore, the antitumor and antimetastatic effects of NOS inhibition, previously attributed, in part, to reduced tumor cell invasiveness,[16] may also be explained by reduced neovascularization.

Inherent NOS activity of the eNOS-expressing mammary adenocarcinoma cells utilized in the present research is likely the major source of NO contributing to the NO-mediated induction of angiogenesis. eNOS expression by endothelial cells, as well as well as iNOS expression by some macrophages and endothelial cells may serve as additional, minor sources of NO in these tumor implants.

The precise molecular mechanisms responsible for reduced angiogenesis with NOS inhibition in our model remain to be determined. NO is required for endothelial cell proliferation, migration and organization, key components of the angiogenic cascade.[22,34,35] Using a rabbit cornea assay, it has been shown that NO is a downstream mediator of VEGF-induced angiogenesis, since it could be blocked by administering L-NAME.[35] Further evidence supports this notion; angiogenesis in response to tissue ischemia (an inducer of VEGF) was reduced in $eNOS^{-/-}$ mice.[36] VEGF-stimulated proliferation of endothelial cells, triggered by NO, was shown to require intracellular signalling via cGMP-dependent protein kinase (PKG),[37] Raf-1 kinase[37] and mitogen activated protein (MAP) kinase.[35] Our preliminary results (data not shown) indicate that the C3L5 mammary adenocarcinoma cells utilized in the present research express VEGF protein *in vitro*. Whether VEGF expression in these cells is induced by endogenous NO remains to be determined; an upregulation of VEGF mRNA by NO was reported for rat mesangial cells.[38] Coexpression of eNOS and VEGF in C3L5 cells may equip them with a dual advantage in inducing NO-mediated angiogenesis from the host vasculature: VEGF-mediated stimulation of NO in the vascular endothelium, and NO produced by tumor cells by activation of eNOS.

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Figure Legends

Figure 1

Gross morphology is shown for tumor-exclusive Matrigel implants (A), and tumor-inclusive Matrigel implants obtained from L-NAME and D-NAME-treated animals (B and C, respectively). Tumor-exclusive implants were translucent and avascular, and unaffected by L-NAME or D-NAME treatment. Tumor-inclusive implants were larger, and those obtained from L-NAME-treated animals were less vascular relative to those obtained from D-NAME-treated animals. Photomicrographs of Masson's Trichrome staining are shown for tumor-exclusive (D), and tumor-inclusive Matrigel implants obtained from L-NAME (E) and D-NAME-treated (F) animals. Tumor-exclusive implants obtained from L-NAME and D-NAME-treated animals were avascular and contained a few fibroblasts. The sections of tumor-inclusive implants in both treatment groups showed three histologically distinct areas, shown in Figure 1G (from L-NAME-treated animal): peripheral stroma (*S*), adjacent tumor-dominant area (*T*), and central zone of necrosis (*N*). The stromal components in L-NAME-treated animals appeared thinner and less vascular relative to those in D-NAME-treated animals. Magnification bars: panels 1A - 1C, 1 mm; panels 1D - 1G, 30 μm.

Figure 2

Immunohistochemical localization of CD31 antigen is shown in top panel in tumor-inclusive implants from D-NAME and L-NAME-treated animals (A and B, respectively), identifying endothelial cells lining the microvasculature within the tumor component of the implants. Neovascularization was reduced in L-NAME-treated animals relative to those treated with D-NAME. Immunohistochemical localization of eNOS antigen is shown in middle panel in tumor-inclusive implants; positive immunostaining and negative control are shown (C and D, respectively). A high proportion of tumor cells and endothelial cells lining the tumor vasculature (inset in C) within implants expressed eNOS, regardless of treatment group. The bottom panel shows immunohistochemical localization of iNOS antigen in peripheral stroma (E) and healthy tumor (F) bordering necrotic area. Expression of iNOS did not differ between treatment groups. Positive immunoreactivity for iNOS protein was evident in macrophages (large arrows) located in stromal (E) and tumor (F), as well as within central necrotic (not shown) areas of the implants in both treatment groups. Endothelial cells (small arrow) stained positively for iNOS, and some nonspecific staining of stromal tissue

was observed in both treatment groups. Magnification bars: panels A - D, 30 μm; panels E - F, 50 μm; inset panel 2C, 60 μm.

Figure 3

Quantification of tumor-induced neovascularization in sections stained with Masson's Trichrome (A and B) and immunostained for CD31 (C and D). The data were expressed as the maximum number of blood vessels per field (mean ± S.E.; n = 15 animals per group) and as the average number of blood vessels in 3 fields of maximal density (mean ± S.E.; n = 15 animals per group). Irrespective of staining protocol or method of quantification utilized, the neovascular response was reduced in L-NAME-treated mice relative to those treated with D-NAME as indicated by the P value in each panel: A: $P < 0.003$; B: $P < 0.001$; C: $P < 0.0099$; D: $P < 0.0009$. BVC = blood vessel count.

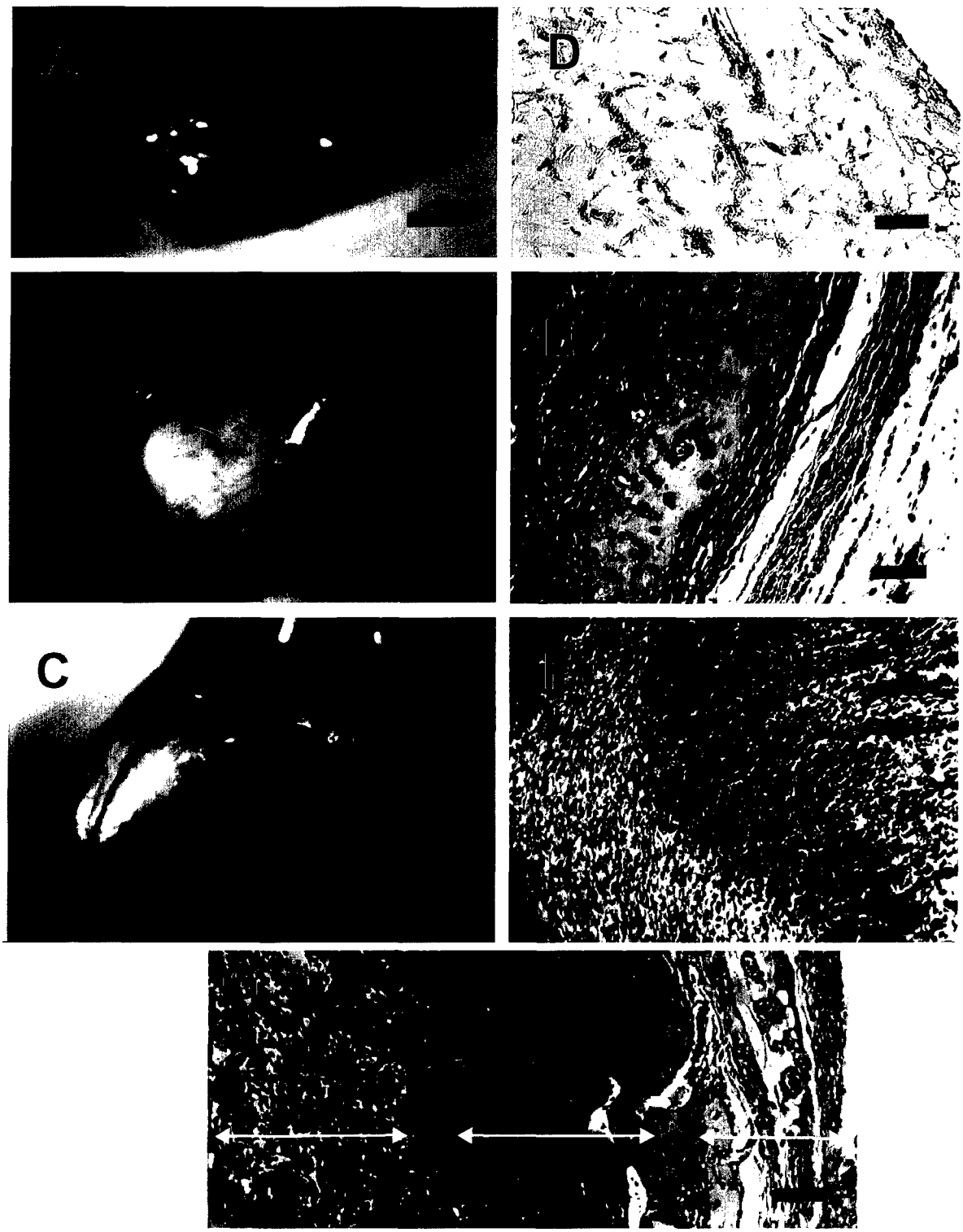


Figure 1

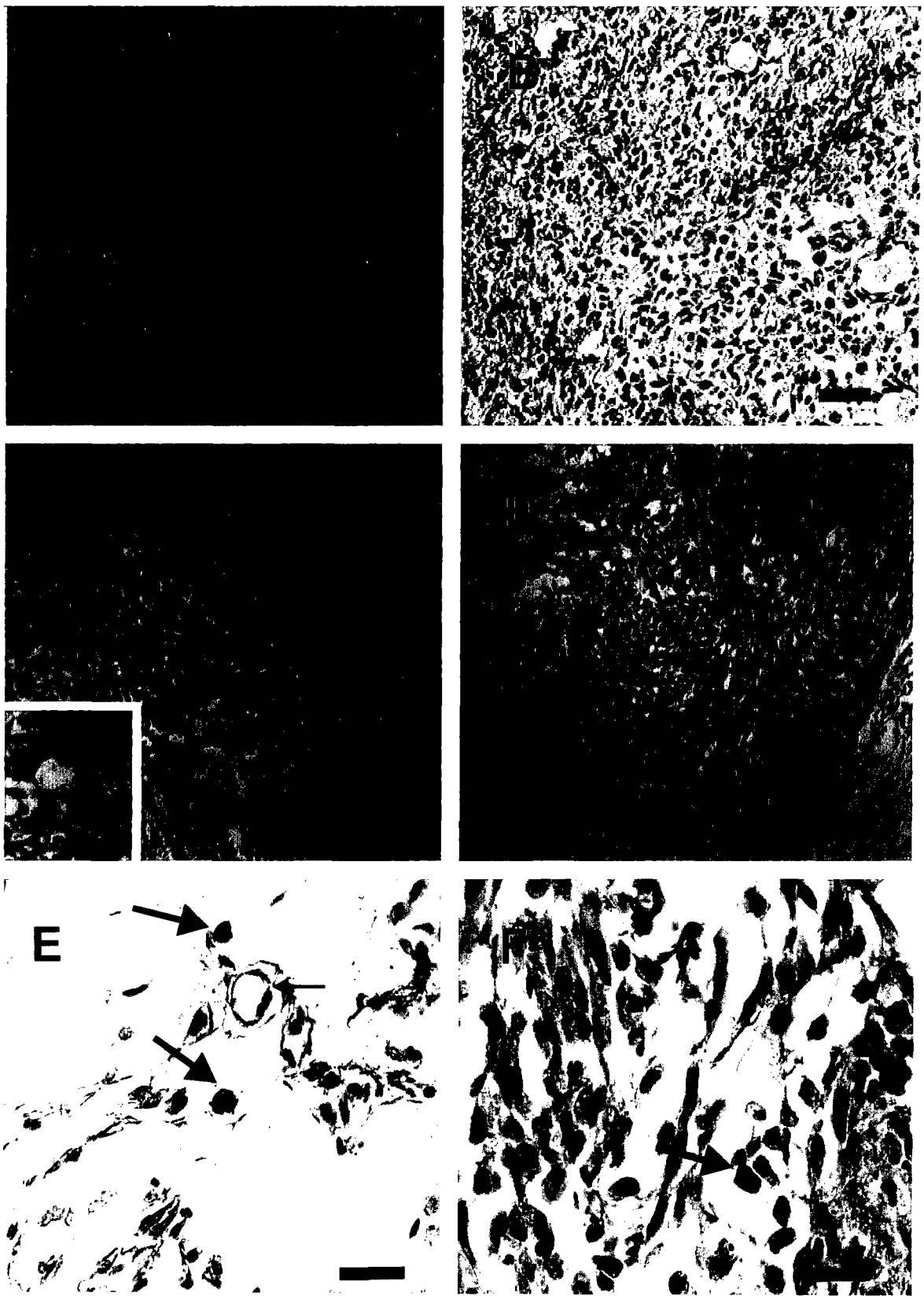
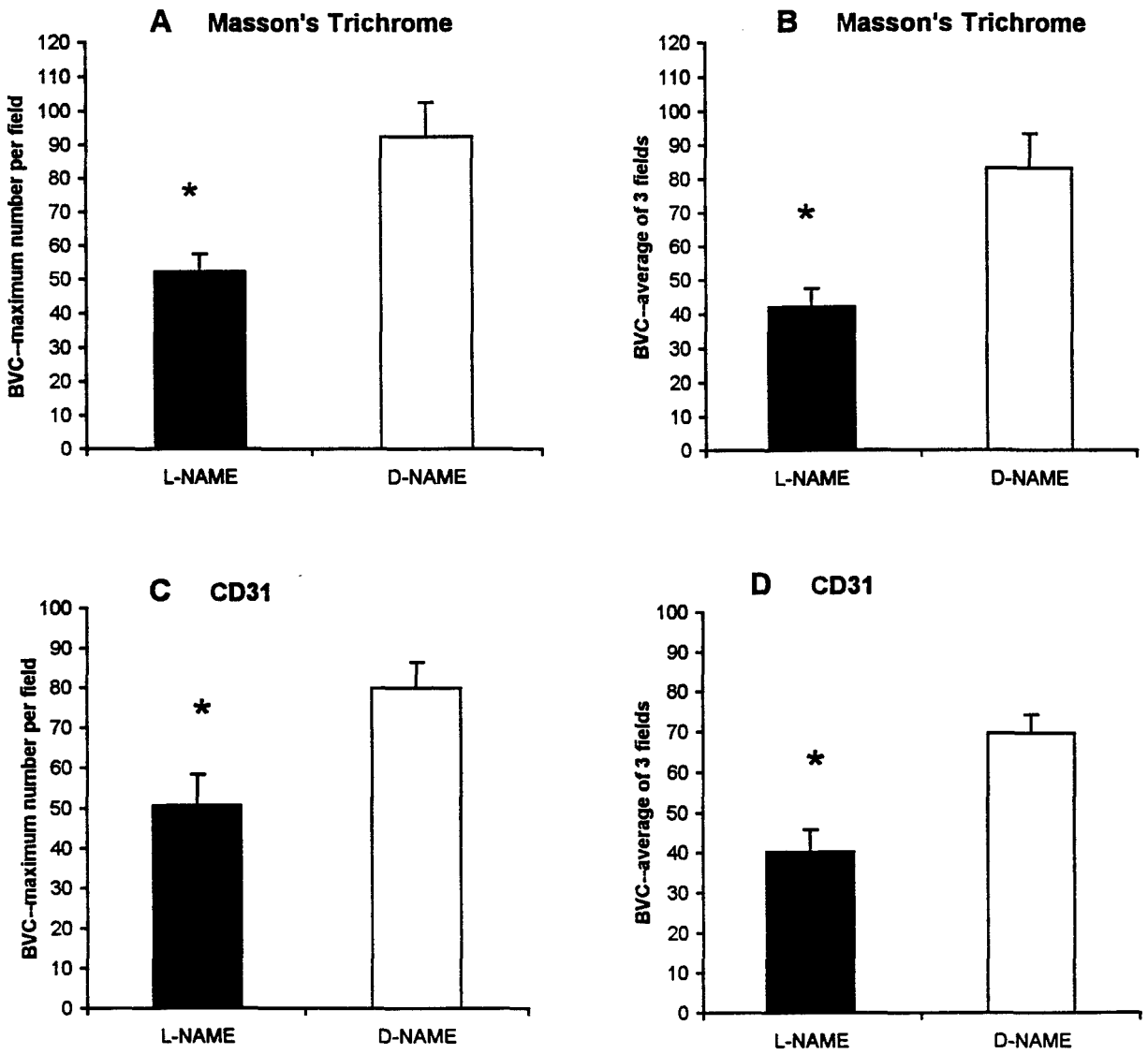


Figure 2


A Masson's Trichrome
BVC--maximum number per field
120
110
100
90
80
70
60
50
40
30
20
10
0
*
L-NAME
D-NAME
B Masson's Trichrome
BVC--average of 3 fields
120
110
100
90
80
70
60
50
40
30
20
10
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L-NAME
D-NAME
C CD31
BVC--maximum number per field
100
90
80
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L-NAME
D-NAME
D CD31
BVC--average of 3 fields
100
90
80
70
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40
30
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10
0
*
L-NAME
D-NAME

Figure 3

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 18 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request for Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grants DAMD17-96-1-6096 and DAMD17-98-2-8019. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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